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A BIOCHEMICAL STUDY OF HUMAN LEUKOCYTE ALKALINE PHOSPHATASE

by

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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.....
Supervisor

.....

.....

DATE Dec. 10, 1981....

to Pooh, Goose

and Billy

ABSTRACT

The hydrolysis of p-nitrophenylphosphate (pNPP) and cysteamine-S-phosphate (CASP) by purified human liver, kidney, neutrophil, small-intestinal mucosa and placental alkaline phosphatase has been studied. The affinity towards CASP was variable amongst the multiple forms of human alkaline phosphatase with K_m values ranging from 0.09 mM to 1.00 mM. The K_m values for pNPP were more consistent and showed a range of 0.017 mM to 0.038 mM. The ratios of the rate of hydrolysis of pNPP to the rate of hydrolysis of CASP were similar amongst the multiple forms of human alkaline phosphatase (1.5 - 1.9) with a mean value of 1.7.

The mean value of the ratios of the rate of hydrolysis of pNPP to the rate of hydrolysis of CASP for thirty-six normal sera was 2.1 ± 0.5. Twenty-five sera from patients with lymphoma, infectious mononucleosis, chronic or acute lymphatic leukemia, or Burkitt's lymphoma displayed a mean ratio of 2.0 ± 0.4. The mean ratio for twenty-eight sera from patients with myeloma, acute myeloid leukemia, lung cancer, sarcomas or Hodgkin's disease was 2.3 ± 0.5. The sera from nine patients with increased serum alkaline phosphatase levels due to pregnancy or other conditions had a mean ratio value of 1.8 ± 0.3. Therefore, no evidence was found of an unique alkaline phosphatase which was incapable of cysteamine-S-phosphate hydrolysis and acted as a marker for lymphoproliferative disorders.

Granulocytes were isolated to 98% purity from whole blood of fifty healthy individuals. Aqueous solutions from butanol extracts of the isolated granulocytes revealed alkaline phosphatase levels of 9.5 mU/10^7 granulocytes to 36.4 mU/10^7 granulocytes with a mean value of 19.4 ± 6.9 mU/10^7 granulocytes. Protein determinations correlated well with the number of granulocytes present.

Inhibitor studies using L-phenylalanylglycylglycine, L-phenylalanine and L-homoarginine revealed a distinct pattern for each of the liver, intestinal and placental isoenzymes of human alkaline phosphatase. Crude extracts and purified enzyme from the same tissue sources showed similar patterns. Aqueous solutions from butanol extracts of human granulocytes displayed a pattern virtually identical to that of the liver alkaline phosphatase which is consistent with the proposal that it is the product of the same structural gene as the liver/kidney/bone group of human alkaline phosphatases.

Therefore, all of the multiple forms of human alkaline phosphatase tested in this study were capable of the hydrolysis of cysteamine-S-phosphate although their affinities toward this substrate were variable. The sera from patients with lymphoproliferative disorders did not contain an unique form of alkaline phosphatase and acted in a similar manner as sera from healthy individuals. The alkaline phosphatase in human neutrophils showed a wide range of activity and was found to be the same gene product as the liver, kidney and bone forms of human alkaline phosphatase.

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LIST OF ABBREVIATIONS

ACTH	adrenocorticotrophic hormone
AMP	2-amino-2-methyl-1-propanol
CASP	cysteamine-S-phosphate
CPD	citrate-phosphate-dextrose
c.v.	coefficient of variation
DTNB	5,5'-dithiobis (2-nitrobenzoic acid)
EDTA	ethylenediamine tetra-acetic acid
Group I	disorders claimed by Neumann et al (1979) to be associated with the presence of N-alkaline phosphatase in the serum
Group II	disorders claimed by Neumann et al (1979) not to be associated with the presence of N-alkaline phosphatase in the serum and disorders which have not been previously studied for the presence of N-alkaline phosphatase
Group III	conditions where patients have increased levels of serum alkaline phosphatase
Hrg	L-homoarginine
Phe	L-phenylalanine
PGG	L-phenylalanylglycylglycine
pNPP	p-nitrophenylphosphate
S.D.	standard deviation
TES	N-tris [hydroxymethyl] methyl-2-aminoethane sulfonic acid
TLC	thin layer chromatography
TRIS	2-amino-2-hydroxy-methylpropane-1,3-diol
$\frac{V_{pNPP}}{V_{CASP}}$	ratio of the rate of hydrolysis of pNPP to the rate of hydrolysis of CASP
$\bar{x}$	mean

CHAPTER ONE

Introduction to Human Leukocyte Alkaline Phosphatase

1. History

The first biochemical demonstration of phosphatase activity in neutrophils was reported by Kay in 1930. The enzyme was further characterized by Roche (1931) who discovered that the enzyme from rabbit, guinea pig and horse white blood cells had a pH optimum of 8.6.

The advent of a histochemical technique, developed in 1939 by two independent workers (Gomori and Takamatsu) resulted in a flurry of studies since it simplified the detection of alkaline phosphatase activity to such a large extent. Gomori's technique involved the hydrolysis of substrate (β-glycerophosphate) to form phosphate ions which reacted with calcium ions in the incubation mixture to form insoluble, microscopically invisible calcium phosphate. When these tissue sections were exposed to cobalt nitrate, cobalt phosphate was formed which reacted with ammonium sulphide to form black, insoluble cobalt sulphide which was readily visible microscopically. The first detailed report of human neutrophil alkaline phosphatase activity in normal and pathological states using Gomori's technique was made by Wachstein in 1946. Unfortunately, phosphatase diffusion was a significant problem with this technique which resulted in prominent nuclear staining and made semiquantitative scoring of the intensity of the reaction product inaccurate. In addition, cytological details were poorly preserved.

A different approach which utilized an azo dye coupling method was outlined by Kaplow (1955). Upon enzymatic hydrolysis of α-naphthyl phosphate, the liberated naphthol immediately coupled with the diazonium salt of 4-benzoyl-2:5-methoxyaniline (Fast Blue RR) to form an insoluble azo dye deposit. Mayer's aqueous hematoxylin was used as a nuclear counterstain. The enzyme activity seemed limited to the cytoplasm of mature neutrophils and occasionally to band neutrophils. A scoring procedure was developed in which 100 consecutive mature neutrophils were rated from 0 to 4 on the basis of the intensity and appearance of the precipitated dye. The sum of the ratings was considered the score for a given blood smear. This technique soon became widely used as a routine hematological procedure and is still popular today.

With the development of techniques which allowed leukocyte separation from whole blood in the early 1950's biochemical studies of isolated white blood cells were made possible. Since then numerous workers have used both cytochemical and biochemical assay methods to determine some of the properties of neutrophil alkaline phosphatase.

2. Properties

Alkaline phosphatase from neutrophils is capable of liberating inorganic phosphorus from a wide variety of substrates including nucleotides (Trubowitz et al, 1961) although not all substrates are hydrolyzed at similar rates. Trubowitz et al (1957) also showed that human leukocyte alkaline phosphatase is predominantly zinc dependent since EDTA clearly inhibited enzyme activity through chelation. Instability of neutrophil alkaline phosphatase to heat

has been reported by several authors (Tangheroni et al, 1971; Diamant et al, 1970; Findlay and Johnston, 1977; Wilson et al, 1981) and numerous reports have been made of the stability of neutrophil alkaline phosphatase in the presence of L-phenylalanine (Tangheroni et al, 1971; Findlay and Johnston, 1977; Wilson et al, 1981). Reports concerning the heterogeneity of human neutrophil alkaline phosphatase have been contradictory. Trubowitz et al (1957) studied the human enzyme and found evidence of two different forms, one of which required magnesium and the other zinc for maximal activation. Conversely a later study (Trubowitz et al, 1961) indicated that, on the basis of data from competitive inhibition experiments, a single enzyme was responsible for the enzymatic activity. Variable reports of two and three electrophoretic bands have further confused the issue (see McComb, Bowers and Posen, 1979 for review). Rosner and Lee (1972) observed that radioactive zinc migrated with a slower-moving form whereas radioactive magnesium migrated with a faster-moving form of the enzyme. Lyons et al (1968) reported the presence of three electrophoretic bands in granulocyte alkaline phosphatase from normal subjects. Moreover, not all subjects displayed all three bands and the pattern of any given individual was variable from one examination to the next. There is no readily apparent reason for the conflicting electrophoretic reports although as indicated by McComb, Bowers and Posen (1979) there is the possibility that the variable bands are due to incomplete separation of alkaline phosphatase from the neutrophil membrane.

3. Mechanism of hydrolysis

A detailed description of the mechanism of hydrolysis by alkaline

phosphatase is available in McComb, Bowers and Posen (1979).

Very briefly, there is now considerable evidence that alkaline phosphatase from E. coli and probably from mammalian tissue sources as well is a dimer consisting of two identical subunits with zinc forming an integral part of the molecule.

The reaction sequence of alkaline phosphatase can be divided into four major steps. The initial step is the formation of a Michaelis complex which is converted to a covalently-bonded phosphoryl-enzyme complex during cleavage of the alcohol. This complex is converted into an enzyme-phosphate-addition complex, followed by terminal dephosphorylation resulting in release of orthophosphate and regeneration of free enzyme.

It is generally agreed that the formation of the Michaelis complex and the scission of the leaving group are not rate-limiting. The evidence is not sufficiently clear, however, to determine whether the formation or breakdown of the covalent complex or conformational changes constitutes the rate-limiting step.

4. Function

The physiological role of alkaline phosphatases remains elusive. A connection with transport, possibly of phosphate, has been suggested based on its high levels in locations where tissues are concerned with active transport such as the adsorptive surfaces of the proximal convoluted tubules in the kidney, the small-intestinal mucosa, the syntrophoblast of the placenta and the cell surface of E. coli (Posen, 1967). It has also been postulated (Fishman, 1974) that alkaline phosphatase may act with other membrane phosphohydrolases and transferases to regulate the dimensions of the membrane.

It is widely accepted that alkaline phosphatase is capable of catalyzing the transfer of the phosphoryl group from phosphomonoesters to hydroxyl-containing acceptor molecules in addition to its much more widely-studied phosphohydrolase activity. The phosphotransferase activity has not undergone much investigation due to the lack of discovery of acceptor molecules with high affinity for the enzyme. This certainly does not however, rule out the fact that the phosphotransferase activity may be important physiologically for the synthesis of phosphomonoesters. It is also possible that alkaline phosphatase may play some kind of a regulatory role since phosphoprotein phosphatase activity has been demonstrated by placental alkaline phosphatase (Huang et al, 1976) and it has been suggested that protein phosphorylation and dephosphorylation is important in connection with metabolic regulation (Rubin and Rosen, 1975).

There have also been suggestions that the role of alkaline phosphatase is related to maintenance of cell shape. Fiskin et al (1980) reported a transient expression of alkaline phosphatase over the surface of HeLa cells which was associated with formation of cell extensions. Although erythrocytes do not contain alkaline phosphatase, a recent report by Lovlin et al (1980) indicated that changes in red cell volume were related to plasma alkaline phosphatase activity. These authors stated that phosphorylation of membrane proteins, particularly the spectrin-actin complex, appears critical for maintenance of the structural integrity of the red blood cell and suggested that plasma alkaline phosphatase may play a role in this respect. Perhaps in a similar fashion neutrophil

alkaline phosphatase is related to the maintenance or changing of cell shape in view of the fact that neutrophils are very mobile, deformable cells which are phagocytic.

A study by Schwender (1981) proposed that antigen-antibody induced calcium uptake into cells could involve alkaline phosphatase in the biochemical mechanism leading to the release of allergic mediators, since a close relationship between alkaline phosphatase and calcium transport had been previously reported (Limas and Cohn, 1973; Russell et al, 1972). Allergic mediator release inhibitors such as cromolyn sodium inhibited alkaline phosphatase which suggested its involvement in antigen-antibody-induced allergic responses. The formation of a phosphoprotein during inhibition of mediator secretion has also been observed, and may be due to the inhibition of alkaline phosphatase. It has been thought that alkaline phosphatase in leukocytes played a role in bactericidal killing (see De Chatelet et al, 1979) but this has been challenged by the description of an apparently healthy individual with markedly abnormal leukocyte alkaline phosphatase levels and normal bactericidal activity of his cells (De Chatelet et al, 1979). In addition, L-p-bromolevamisole markedly inhibited alkaline phosphatase of normal leukocytes but had no effect on the ability of intact cells to kill bacteria. Considerable efforts are being directed toward the elucidation of the physiological role of human alkaline phosphatase.

5. Intracellular location

Alkaline phosphatase activity within neutrophils appears to be located within the cytoplasm when an azo dye-coupled cytochemical technique is employed (Kaplow, 1968). Cytochemical methods used

with electron microscopy showed that alkaline phosphatase activity appeared first during the myelocyte stage and was connected with immature specific granules (Bainton et al, 1971). Conversely, using a similar technique, Borgers et al (1978) reported that specific granules were not reactive and that alkaline phosphatase activity was confined to the external part of the plasma membrane. Subcellular fractionation studies of human neutrophils (West et al, 1974; Bretz and Baggiolini, 1974; Spitznagel et al, 1975) revealed that the enzyme was localized to a fraction containing a heterogeneous collection of membranes derived from plasma membranes, mitochondrial outer membranes, the Golgi and the endoplasmic reticulum. In 1979, Rustin et al further resolved this heterogeneous fraction and, complemented by electron microscopy experiments, localized alkaline phosphatase to a unique organelle in human neutrophils. They have confirmed these findings in later studies and have now termed the unique organelles phosphasomes (Rustin and Peters, 1979; Wilson et al, 1981). A study by De Pierre and Karnovsky (1974) of guinea pig neutrophils demonstrated that alkaline phosphatase was an ectoenzyme since pNPP was hydrolyzed by intact cells. However since there was twice as much enzyme activity after disruption of the cells the presence of enzyme in granules could not be entirely ruled out. It is possible that alkaline phosphatase is present on the membrane of neutrophils as an ectoenzyme and that phosphasomes are the result of invagination and pinching off of the plasma membrane to form vesicles.

6. Levels relating to cell maturity

There is controversy within the literature as to what relation-

ship the level of alkaline phosphatase within neutrophils has to the age of the cells. Trubowitz et al (1959) found that when measured both biochemically and histochemically the level of alkaline phosphatase in bone marrow granulocytes was approximately 50% lower than that of the cells in peripheral blood. They suggested that young cells fresh from the marrow have lower levels of alkaline phosphatase and synthesize additional enzyme during their life span in the peripheral blood. On the basis of electron microscopy studies in conjunction with cytochemical stains, Bainton et al (1971) reported that alkaline phosphatase activity first appeared during the myelocyte stage and was associated with specific granules. Whereas the number of azurophilic granules per cell was reduced by mitosis during the myelocyte stage, specific granules were formed continually throughout this stage and progressively accumulated. Fehr and Grossman (1979) investigated the alkaline phosphatase activity of the circulating and marginated pool of intravascular neutrophils using both cytochemical and biochemical assay methods. Upon administration of epinephrine, which caused temporary demargination, an increase in activity was seen. Conversely hydrocortisone, which is known to cause granulocyte mobilization from the bone marrow reserve, caused a decrease in the level of enzyme activity. They concluded that functionally or chronologically older neutrophils have higher alkaline phosphatase activity and that transfer from the circulating to marginated pool is a selective rather than random process. In an investigation by Bondue et al (1980) myeloid precursors were labelled in vivo with tritiated thymidine and allowed to mature into marrow neutrophils. An injection of cortisol caused premature marrow

release which revealed that labelled cells had approximately 50% less alkaline phosphatase than unlabelled cells when measured cytochemically. These authors concluded that neutrophil alkaline phosphatase levels increased with cellular age.

An opposing viewpoint was presented by Spiers et al (1975). Using a cytochemical method of assay they found that, upon removal of the spleen, the level of neutrophil alkaline phosphatase rose. Their explanation of this phenomenon was that young cells, normally sequestered in the spleen, were being released into the circulation. In other words newly-formed cells had high levels of alkaline phosphatase activity which decreased as the cell matured. However Kaplow (1976) suggested that the increase in levels witnessed by Spiers et al was simply a response to the surgical trauma of splenectomy. On the other hand, a report by Dallegri et al (1979) supported the hypothesis of Spiers et al (1975) since they found that newly-formed neutrophils of chronic myelogenous leukemia grown in liquid culture were normal as far as neutrophil alkaline phosphatase activity was concerned using Kaplow's cytochemical method. They suggest that the low levels of neutrophil alkaline phosphatase usually seen in chronic myeloid leukemia are due to accumulation of aged alkaline phosphatase-negative neutrophils. Mishler and Williams (1980) compared peripheral blood levels of neutrophil alkaline phosphatase in healthy volunteers before and after a challenge of aetiocholanolone or prednisolone, both of which are known to release neutrophils from the bone marrow reserve. They found an increase in alkaline phosphatase levels concommitant with an increase in the number of immature neutrophils and concluded that alkaline phosphatase levels

in neutrophils decrease with increasing age.

A similar situation was found in rats by Williams in 1975 who reported a highly significant negative correlation between the age and the alkaline phosphatase levels of the neutrophils. In addition, levels of alkaline phosphatase rose in the circulating neutrophils of rats under conditions where a cell population younger than normal was present (Williams and Johnson, 1976). In 1978, Williams et al found higher levels in bone marrow neutrophils than in circulating neutrophils in rats.

It is evident from these conflicting reports that further investigation is required to establish what the relationship is, if any, between cellular levels of alkaline phosphatase and the age of the cell.

7. Neutrophil alkaline phosphatase levels in the normal population

The ontogeny of human neutrophil alkaline phosphatase was investigated by Kelemen et al (1978) using the cytochemical method of Hayhoe and Quaglino (1958). They reported that embryonic neutrophils in the liver and early bone marrow neutrophils were negative for alkaline phosphatase activity during the first trimester of pregnancy. Occasional cells with a strong neutrophil alkaline phosphatase reaction appeared during the second trimester and activity increased greatly in the third trimester. Term babies had slightly higher than normal neutrophil alkaline phosphatase activity in their circulating cells. O'Kell (1968) used Kaplow's (1955) cytochemical method and reported elevated levels in the infant at birth, a slow decline during the first five days of life, and a rapid fall from the fifth to tenth day of life with normal childhood levels being

reached by the fifteenth day. This pattern of alkaline phosphatase levels was similar to that of estrogens and progesterone. Sadovsky et al (1975) used the same cytochemical method and noted a significant decrease in the neutrophil alkaline phosphatase activity of newborns on the fourth day after delivery.

Using a biochemical assay method Rosner and Lee (1968) showed that children have higher levels of neutrophil alkaline phosphatase than adults do and that levels gradually decrease towards puberty. No differences were apparent between the sexes in children, but significant differences were found between men and women. Women displayed average levels which were 50% higher than those of men, although after menopause the values for women approached the values for men (Rosner and Lee, 1968).

A sudden cytochemically-detected increase in neutrophil alkaline phosphatase levels in the middle of the menstrual cycle was noted by Gordon and Hunter (1965). This finding was confirmed by Diamant and Polishuk who in 1979 reported that ovulation occurred when neutrophil alkaline phosphatase levels had reached a value five times that of the basal level when detected cytochemically. They claimed that estrogen caused an increase in level which was later counteracted by progesterone. A biochemical study of the enzyme by Crook et al (1980) detected diurnal variation of neutrophil alkaline phosphatase activity in three normal males.

Other hormonal effects on neutrophil alkaline phosphatase have been detected biochemically. Elevation of levels occurred in response to ACTH and adrenal steroids (Valentine et al, 1957). Rosner and Lee (1965) demonstrated that androgenic hormones inhibited

the enzyme. They also suggested that growth hormone may have a possible stimulating role since the very high levels of enzyme found in young children gradually decrease with the approach of puberty.

Thus, with so many factors affecting the levels of neutrophil alkaline phosphatase normally present and so many differences in methods of detection and expression of results, it is virtually impossible to quote a universally-accepted biochemically or cytochemically-detected reference range. Perhaps the best summation of the situation is to say that the observations made by Hayhoe and Quaglino back in 1958 still hold true today: there is a large normal range of neutrophil alkaline phosphatase activity with no clear relationship between the level of positivity and the leukocyte count. However, once one has established which method of detection is to be used and what the reference range is for that method, the test has several useful clinical applications. This aspect is discussed in a later section of this chapter.

8. Medical conditions associated with abnormal levels of neutrophil alkaline phosphatase

Although Valentine et al (1954) mentioned that neutrophil alkaline phosphatase was increased in four gravid women, Pritchard (1957) is generally given credit for the discovery of significantly increased neutrophil alkaline phosphatase levels during pregnancy. Using a cytochemical assay he found that an increase usually appeared during the first trimester, with levels reaching a plateau during the second trimester and rising abruptly during labour and the early post-partum period. This was confirmed by Quigley et al (1960) who,

using Kaplow's method, also noted that high levels were maintained throughout the first post-partum week but decreased back to normal during the next six weeks. The mechanism for this increase during pregnancy is not known although it has been suggested that it is related to the increased production of corticosteroids during normal pregnancy (Pritchard, 1957). It has also been shown that this increase in the level of neutrophil alkaline phosphatase is independent of the placental form of alkaline phosphatase which appears in the serum during pregnancy (Diamant et al, 1970; Findlay and Johnston, 1977).

It is also well documented that levels of neutrophil alkaline phosphatase are increased in cases of pyogenic infection (Wachstein, 1946; Valentine and Beck, 1951; Wiltshaw and Moloney, 1955) although the reason for the increase remains obscure. As mentioned earlier, although it was at one time thought that alkaline phosphatase was involved with bactericidal activity, this view has been challenged by De Chatelet et al (1979). Contrary to the case of adults, Donato et al (1979) found low neutrophil alkaline phosphatase activity in all infected newborn infants.

Many hematological disorders have been associated with changes in leukocyte alkaline phosphatase levels. Increased levels have been reported to occur in polycythemia rubra vera and myelofibrosis with myeloid metaplasia (Mitus and Kiossoglou, 1968 and references therein) and in Down's Syndrome (see Tangheroni et al, 1971). Furthermore, the increase in activity in these cases is not related to qualitative changes in the enzyme (Rosenblum and Petzold, 1973; Tangheroni et al, 1971). Decreased levels have been reported in

pernicious and aplastic anemia (Rosner and Lee, 1965), a case of monocytic leukemia (Garg and Silber, 1972), a case of hairy cell leukemia (Zeya et al, 1979) and in classic haemophilia and Von Willebrand's disease (Jankovic, 1979). Grozdea et al (1980) disputed Jankovic's results and found that age affected the neutrophil alkaline phosphatase score and that some haemophiliacs with no treatment had high scores. An early-discovered (Wachstein, 1946) and intensely-studied decrease in neutrophil alkaline phosphatase is also found in chronic myeloid leukemia. It has now been established that in this condition there is not an abnormal form of the enzyme present but rather a decrease in the level of the normal enzyme protein (Rustin and Peters, 1979; Wilson et al, 1981). It has also been shown in two independent reports that an extrinsic factor controls neutrophil alkaline phosphatase synthesis in chronic myeloid leukemia since cells from patients with this condition and low alkaline phosphatase activity developed an increase in activity when injected into individuals who were not suffering from this condition (Schiffer et al, 1979; Rustin et al, 1980). Hellman and Goldman (1980) also supported this concept of external modulating influences on neutrophil alkaline phosphatase since they found cultured chronic myeloid leukemia neutrophils had high levels of alkaline phosphatase activity.

9. Clinical applications

By far the most widely used clinical application of neutrophil alkaline phosphatase levels is as an aid in differentiating between leukemoid reactions and chronic myeloid leukemia since in these two conditions the peripheral blood picture is similar. Usually a cytochemical stain is employed which reveals elevated alkaline phos-

phatase levels in a leukemoid reaction or substantially reduced levels in chronic myeloid leukemia.

More recently Diamant and Polishuk (1979) have advocated the use of the leukocyte alkaline phosphatase cytochemical stain to indicate ovulation. They reported successful conception in six women who were artificially inseminated on the day when the leukocyte alkaline phosphatase relative score reached a value of five times the basal level.

Grozdea et al (1981) reported that levels of granulocyte alkaline phosphatase in blood smears which were stable to heat (65°C for 10 min) rose during the first trimester of a normal pregnancy. By contrast, spontaneous abortion during the first trimester was accompanied by a drop in the level of thermostable alkaline phosphatase. They recommended that this technique be used to monitor women with a history of repeated spontaneous abortions since a rising level of thermostable alkaline phosphatase seemed to indicate a favourably progressing pregnancy whereas a drop in activity seemed to indicate an approaching miscarriage.

Donato et al (1979) indicated that low leukocyte alkaline phosphatase activity in newborns may be helpful in the diagnosis of neonatal bacterial infections. Studying an adult population Mackie et al (1979) concluded that although mean leukocyte alkaline phosphatase levels were significantly elevated over normal in cases of systemic and localized infection there was considerable overlap of individual scores in each patient group which severely limited the usefulness of the technique for the diagnosis of infection in any individual.

Harper and Quigley (1961) suggested the use of a cytochemical leukocyte phosphatase stain for simple and rapid detection of preg-

nancy. They reported positive results very early after fertilization and claimed that the accuracy was comparable to commonly employed pregnancy tests based on chorionic gonadotrophin. This usage never became popular, presumably because of the non-specificity of the test.

10. Alkaline phosphatase activity in leukocytes other than neutrophils

Many studies using cytochemical methods have reported that lymphocytes displayed no alkaline phosphatase activity (Kaplow, 1968; Timperley and Ahmed, 1970; Nanba et al, 1977) and low levels of activity detected biochemically in lymphocyte suspensions were attributed to contamination by neutrophils (Tittobello and Agostoni, 1967). In 1969, Kaplow reported the cytochemical detection of weak to moderate alkaline phosphatase activity in a small percentage (0 - 1.5%) of normal peripheral blood lymphocytes. No significant differences were found between adults and children, males and females, or healthy and hospitalized individuals. A study by Ruuskanen et al (1975) investigated alkaline phosphatase activity ultracytochemically in guinea pig thymocytes. They reported alkaline phosphatase activity was a feature of immature T cells, and maturation was associated with a loss in enzyme content so that alkaline phosphatase activity was not seen in circulating T cells. Using a biochemical assay, Kramers et al (1978) detected measurable activity in normal peripheral blood lymphocytes but their granulocyte contamination ranged from 1 to 10%. They also detected higher levels in cord blood which contains many non-T and non-B lymphocytes but few T lymphocytes. A later study (Foa et al, 1979) fractionated normal

human lymphocytes and found most alkaline phosphatase activity associated with the fraction which had low numbers of T lymphocytes and high numbers of non-T, non-B lymphocytes. Perhaps low levels of alkaline phosphatase are normally associated only with non-T, non-B lymphocytes and since these cells constitute only a small proportion of the normal circulating lymphocyte population the negative results obtained with most cytochemical methods of assay are readily explained. Increased levels of alkaline phosphatase in lymphocytes have been detected in certain lymphatic neoplasms. This point will be elaborated upon in the following section.

The granules of eosinophils from several species have been shown to contain alkaline phosphatase. However, studies of human eosinophils have been few due to the difficulty of obtaining adequate cell numbers and purity. Cao et al (1973) reported that the chemical and physical properties of eosinophil alkaline phosphatase were similar to those of neutrophils but their cell population of eosinophils was described as "rather pure". West et al (1975) isolated the granules from eosinophils of patients who exhibited eosinophilia and reported they totally lacked alkaline phosphatase activity. Williams et al (1978) demonstrated that alkaline phosphatase activity was present on the external membrane of rat eosinophils, and not in the cell's distinctive red granules. Perhaps alkaline phosphatase, if present at all, in human eosinophils is located on the plasma membrane. This would explain the findings of West et al (1975). In any event, alkaline phosphatase activity due to eosinophils is relatively insignificant in the normal human leukocyte population because eosinophils constitute such a minor proportion of the white cells present.

II. Alkaline phosphatase in malignancies

The ability of tumour cells to produce certain types of alkaline phosphatase which subsequently appear in the serum is now widely appreciated. Several examples of this phenomenon are reviewed by Fishman (1974). Perhaps the most widely known example is the "Regan isoenzyme" which has properties similar to the placental isoenzyme of alkaline phosphatase. It has been suggested that synthesis of placental-like alkaline phosphatase by malignant cells is the result of derepression of the structural gene locus which is responsible for the production of the placental enzyme (Fishman et al, 1968).

There have been several reports of increased levels of alkaline phosphatase in neoplastic lymphoid cells in mice (Metcalf et al, 1962; Lumb and Doell, 1970; Neumann et al, 1970; Haran-Ghera et al, 1972).

A similar situation has been reported in humans by Neumann et al (1976) who claimed that an alkaline phosphatase with unique catalytic properties was present in human lymphoblastoid cell lines and leukemic cells. A later report (Neumann et al, 1979) claimed that this enzyme was also found in the sera of patients with lymphoproliferative disorders. The existence of a marker enzyme for these conditions would be extremely useful diagnostically in order to establish whether cells have a lymphoid or myeloid origin. It would also have therapeutic value since it could be used to follow the course of treatment. Nanbe et al (1977) identified alkaline phosphatase activity only with a restricted class of B-cell lymphomas whereas Poppema et al (1981) reported alkaline phosphatase in both normal and neoplastic lymphocytes of B-cell lineage.

It has not been clearly established whether the alkaline phosphatase of normal lymphocytes and that present in lymphomas are the same form of the enzyme although Damle et al (1979) reported that the alkaline phosphatase which they detected in hemopoietic tumours was a "heat-labile Regan type of alkaline phosphatase".

It is evident from this discussion that further investigation is also required in order to elucidate the presence and properties of alkaline phosphatase in lymphoproliferative disorders.

There have been suggestions that alkaline phosphatase activity in neutrophils is increased over normal in cancer patients (Walach et al, 1981) and further increased in patients who have metastatic spread. Ho et al (1979) also studied neutrophil alkaline phosphatase in patients with malignancies but found an increase over normal only in Hodgkin's disease. However, Levine et al (1966) and Lokich (1977) reported decreased levels in malignant disease. Once again, further investigation must be awaited to clarify the issue.

12. Purposes of this investigation

The purposes of this investigation were basically two-fold. Initial efforts were directed towards study of the hydrolysis of cysteamine-S-phosphate by multiple forms of human alkaline phosphatase. Once it was established that human forms of alkaline phosphatase were capable of this type of hydrolysis, a study of sera from patients with lymphoproliferative disorders was undertaken in an attempt to establish the presence of a unique marker alkaline phosphatase in these types of disorder. Later efforts involved the isolation and biochemical quantification of granulocyte alkaline phosphatase in a normal population. Further studies were

carried out to establish whether the enzyme in granulocytes is a new type or is one of the three established gene products of alkaline phosphatase.

CHAPTER TWO

Materials and General Methods

A. MATERIALS

Unless stated otherwise, chemicals were purchased from Fisher Scientific Co. (Fairlawn, N.J.) and biochemicals from Sigma Chemical Co. (St. Louis, Mo.). All compounds were generally of the highest purity available.

The compound 2-bromoethylammonium bromide was purchased from Eastman Kodak Co. (Rochester, N.Y.). Sodium thiophosphate was a product of Alfa Division, Ventron Corp. (Danvers, Ma.).

Cysteamine-S-phosphate (CASP) was synthesized according to the method of Akerfeldt (1959). The purity of CASP was tested by thin layer chromatography on precoated silica gel TLC sheets produced by E. Merck Ag. (Darmstadt, Germany).

A working solution of 5,5'-dithiobis(2-nitrobenzoic acid) was prepared by addition of the acid to a solution of 25 mM TES, pH 7.5, followed by very slow addition of NaOH by means of an automated syringe. This procedure enabled the DTNB to go into solution without extensive hydrolysis occurring.

Sodium dodecyl sulphate was a product of Bio-Rad Laboratories (Richmond, Ca.). Zap-oglobin and Isoton were purchased from Coulter Electronics of Canada Ltd. (Oakville, Ontario).

Purified alkaline phosphatase from bovine intestine was a product of Sigma Chemical Co. (St. Louis, Mo.). Human alkaline phosphatases

obtained from liver, kidney, small-intestinal mucosa and neutrophils were purified by the method of Seargeant and Stinson (1979a) with minor modifications. Partially purified alkaline phosphatase from human placenta (19 µmol/min/mg) was obtained from Calbiochem-Behring Corp. (La Jolla, Ca.) and further purified (Seargeant and Stinson, 1979a). All enzyme preparations were stored at 4°C in 10 mM TRIS/HCl, pH 7.6, which contained 1.0 mM $MgCl_2$ and 0.1 mM $ZnCl_2$.

Fresh whole blood units were supplied by the Red Cross Blood Transfusion Service (Edmonton). Heparinized Vacutainer tubes were purchased from Becton Dickson Canada (Mississauga, Ont.). White cell isolation and separation included the use of Dextran T500 purchased from Pharmacia Fine Chemicals (Uppsala, Sweden) and Lymphoprep, a product of Nyegaard and Co. (Oslo, Norway). Serum samples were obtained from the University of Alberta Hospital and the W.W. Cross Cancer Institute of Edmonton, Alberta and were stored frozen at -20°C until assayed.

All water used was deionized and double distilled; glass distillation constituted the final step. The pH of all buffers was adjusted with HCl or NaOH solutions.

B. GENERAL METHODS

1. Determination of the purity of cysteamine-S-phosphate

Thin layer chromatography on precoated silica gel TLC plates was performed to test the purity of CASP. A solvent system which consisted of ethanol:pyridine:H_2O (10:5:8) followed by a spray of 1% (w/v) $CuCl_2$:50% (v/v) ethanol:5% (v/v) NH_3 was used as described by Dulis and Wilson (1978). A single black spot indicative of CASP was the

result.

2. Alkaline phosphatase assays using p-nitrophenylphosphate as substrate

Assays were generally performed as described by Bowers and McComb (1966) at 30°C in either a Beckman Acta CIII or Cary Model 16 spectrophotometer which was equipped with a temperature-controlled cuvette holder and scale expansion. After a 10 min incubation of enzyme in 0.78 M AMP buffer, pH 9.0, which contained 1.5 mM $MgCl_2$ the reaction was initiated by addition of substrate, producing an assay medium of 10.0 mM pNPP. The rate of product formation was monitored continuously at 404 nm and linear reaction-rate curves were obtained. Enzymic activity was calculated from these curves based on a molar extinction coefficient of 18,700 (1 cm lightpath) for p-nitrophenol (Bowers and McComb, 1966). One unit of enzymic activity corresponds to one μmole of substrate hydrolyzed per min.

3. Alkaline phosphatase assays using cysteamine-S-phosphate as substrate

Assays were generally performed as outlined by Neumann et al (1979) in which cysteamine released through hydrolysis of CASP by the enzyme, is allowed to react with a sulfhydryl reagent, DTNB, to produce 5-thio-2-nitrobenzoic acid (Ellman, 1959). All measurements were performed at pH 9.0 by necessity, due to the overwhelming spontaneous hydrolysis of DTNB at a more alkaline pH. After a 10 min incubation of the enzyme in 0.78 M AMP buffer, pH 9.0, which contained 1.5 mM $MgCl_2$ at 30°C, the reaction was initiated by addition of the substrate, CASP, followed by the immediate addition of DTNB. This resulted in an assay medium composed of 5.0 mM CASP and 0.4 mM DTNB. The rate of

product formation was monitored continuously at 412 nm by either a Beckman Acta CIII or Cary Model 16 spectrophotometer equipped with a temperature-controlled cuvette holder and scale expansion. Due to the spontaneous hydrolysis of both CASP and DTNB, as well as the nonspecific reaction of DTNB with sulfhydryl groups of proteins, it was necessary to determine the rate of increase in absorbance of the following three blanks:

a) DTNB in AMP buffer

b) DTNB and CASP in AMP buffer

c) DTNB and sample in AMP buffer

In all cases linear reaction-rate curves were obtained at 412 nm. The increase in absorbance due solely to CASP hydrolysis by the alkaline phosphatase in the sample was then obtained by subtracting the amount due to the spontaneous hydrolysis of CASP ($\Delta b - \Delta a$) as well as the amount due to the nonspecific reaction with serum proteins and spontaneous hydrolysis of DTNB (Δc) from the total amount obtained from the test system of DTNB, CASP and sample in AMP buffer. An example of the type of tracings obtained appears in Chapter Four, Figure 4. Enzymic activity was calculated on the basis of a molar extinction coefficient (1 cm lightpath) for 5-thio-2-nitrobenzoic acid of 13,600 (Ellman, 1958). One unit of enzymic activity is equal to the hydrolysis of one µmole of substrate per min.

4. Leukocyte isolation and separation

When whole blood units were obtained from the Red Cross Blood Transfusion Service, the majority of red blood cells were removed by sedimentation for 2 hr at 4°C in the presence of 6% (w/v) Dextran in 0.9% (w/v) NaCl. The leukocyte-rich plasma was concentrated further

by centrifugation. A hand-operated tissue grinder was used to break up any cell clumps and form a smooth suspension which was then layered onto Lymphoprep and centrifuged at 800 x g for 20 min. The resulting granulocytic and mononuclear cell layers were removed and resuspended in 10.0 mM TRIS/HCl, pH 7.6, which contained 1.0 mM $MgCl_2$ and 0.1 mM $ZnCl_2$.

Alternatively, 10 mL of whole blood was drawn from a Red Cross whole blood unit into a heparinized Vacutainer tube. After Dextran sedimentation at 4°C, the leukocyte-rich plasma was layered directly onto Lymphoprep, and treated as outlined above.

5. Butanol extraction procedure for leukocyte suspensions

Leukocyte suspensions were mixed on ice or at 4°C for 30 min. Triton X-100 was then added to a concentration of 1% (v/v), and mixing continued for a further 30 min. A volume of 1-butanol which had been cooled to -20°C, equal to three quarters of the cell suspension volume was then added slowly at 4°C. After thorough mixing and centrifugation at 9000 x g at 4°C for 30 min, the aqueous layer was removed and frozen at -20°C until assayed.

6. Protein determinations

The protein content of cell butanol extracts was determined using the Markwell modification of the Lowry procedure (Markwell et al, 1978). Solutions of bovine serum albumin were used to prepare a standard graph. Absorbance readings at 660 nm were made in a Beckman Acta CIII spectrophotometer.

CHAPTER THREE

Cysteamine-S-Phosphate Hydrolysis by Alkaline Phosphatases

A. INTRODUCTION

1. General

Alkaline phosphatase is a hydrolase which has a low specificity and which therefore can act upon a wide variety of organic phosphate esters. Usually, however, its action is limited to monoesters of orthophosphoric acid.

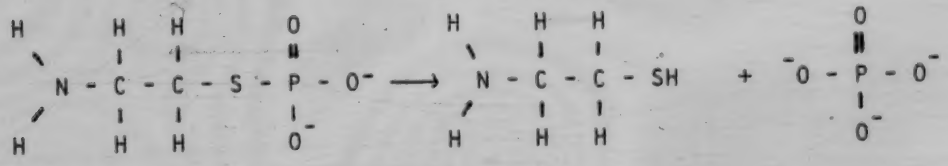
In the mid-1960's the hydrolysis of S-substituted monoesters of phosphorothioic acid by phosphatases present in erythrocyte preparations was noted by Korman et al (1965). A few years later, detailed studies of this kind of hydrolysis were performed using purified enzyme preparations obtained from Eschericia coli (Neumann, Boross and Katchalski, 1967) and from chicken intestine (Neumann, 1968).

With purified enzyme preparations from human tissue sources now available it is of interest to investigate the behaviour of various multiple forms of human alkaline phosphatase with regard to the two classes of substrate mentioned above.

2. Substrate structure

The substrate used to represent the class of O-substituted monophosphate esters of inorganic phosphate was the widely known p-nitrophenylphosphate. Upon enzymatic hydrolysis at an alkaline pH, the phosphate group is cleaved resulting in a highly-coloured nitrophenolate ion which is easily measured spectrophotometrically as outlined in Chapter Two.

The substance used as an example of an S-substituted monoester of phosphorothioic acid was cysteamine-S-phosphate. Hydrolysis by alkaline phosphatase occurs at the S-P bond (Neumann et al, 1967) resulting in the formation of cysteamine and inorganic phosphate as shown below.



Cysteamine-S-phosphate Cysteamine Inorganic Phosphate

In the kinetic assay system employed, the liberated -SH group of the cysteamine reacts with a sulfhydryl reagent, DTNB, under first order conditions. This results in the formation of 5-thio-2-nitrobenzoic acid which is highly coloured and can be measured spectrophotometrically as outlined in Chapter Two.

B. MATERIALS AND METHODS

Preparations of alkaline phosphatase were used which had been extracted and purified essentially as described by Seargeant and Stinson (1979a) from the following human tissue sources: liver, kidney, small-intestinal mucosa, placenta and neutrophils. This selection includes at least one representative of each of the presently-known human alkaline phosphatase isoenzymes. Enzyme prepared from calf intestine was also studied.

Using each substrate, a Michaelis constant for each pure multiple form of alkaline phosphatase was determined from a double reciprocal plot (Lineweaver and Burk, 1934). Substrate concentra-

tions ranging from 0.10 mM to 1.00 mM CASP and 0.005 mM to 0.100 mM pNPP, each in 0.78 M AMP buffer, which contained 1.5 mM $MgCl_2$ were employed for all enzymes except the placental form which required CASP concentrations ranging from 0.04 mM to 0.50 mM. The rate of enzymatic hydrolysis in each case was measured as outlined in Chapter Two, using a Beckman Acta CIII spectrophotometer. Measurements were repeated at least once with good agreement.

Mixed substrate experiments were performed at 30°C by measuring the inhibition of pNPP hydrolysis due to CASP using a buffer system of 0.78 M AMP, pH 9.0, which contained 1.5 mM $MgCl_2$. The hydrolysis of pNPP was monitored continuously at 404 nm at concentrations of 30 µM and 150 µM in the absence of and the presence of at least five different concentrations of the alternate substrate. The concentrations of CASP used ranged from 0.25 mM to 2.00 mM in all cases except for the placental enzyme where concentrations from 0.05 mM to 0.75 mM were required. Dixon plots were prepared from the data and apparent K_i values determined (Dixon, 1953). Measurements were repeated at least once with good agreement.

C. RESULTS

Double reciprocal plots were prepared for each multiple form of alkaline phosphatase using pNPP as substrate. An example of such a plot is shown in Figure 1. A linear relationship was obtained in all cases, and a K_m value for each multiple form was established from the X-intercept of the appropriate graph. Results obtained when measuring CASP hydrolysis were also good and therefore treated in a similar

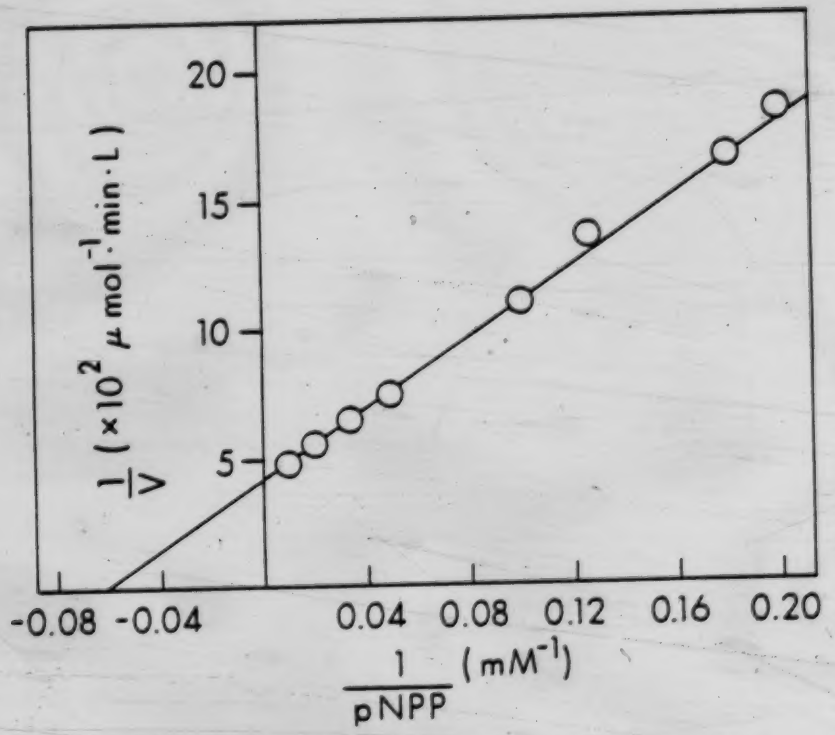


Figure 1. Determination of a Michaelis constant for human placental alkaline phosphatase utilizing pNPP as substrate

Double reciprocal plot obtained for alkaline phosphatase purified from human placenta. Hydrolysis of pNPP was measured at 404 nm in 0.78 M AMP buffer, pH 9.0, which contained 1.5 mM $MgCl_2$ as outlined in the Methods section of this chapter. Similar plots were obtained for all other multiple forms of human alkaline phosphatase tested.

manner, an example of which appears in Figure 2.

In the case of the mixed substrate experiments, Dixon plots were prepared as shown in Figure 3. The pattern obtained is indicative of competitive inhibition and was found in all cases studied. A value for K_i was obtained from the reading of the X-axis at the point of intersection of the two plotted lines. Due to the fact that CASP was not acting strictly as a competitive inhibitor, but rather as an alternate substrate, the K_i values established using this method were termed apparent K_is. This point will be expanded upon in the Discussion section of this chapter.

The K_m values using both substrates, along with the K_i values for all of the multiple forms of alkaline phosphatase studied are compiled in Table I. Examination of the data reveals that although the multiple forms of human alkaline phosphatase have a similar affinity for pNPP, the affinity for CASP varies significantly amongst them. However, a general trend is still apparent, in that for both substrates the small-intestinal mucosa K_ms are the highest, the placental K_ms the lowest, while the others are intermediate between the two. The K_i values also reflect this trend and similarly show a magnitude of ten difference between the highest and lowest value, although the actual K_i and K_m values of CASP of each multiple form are not equal.

In addition, it is apparent that only in the case of the calf intestinal enzyme are the K_m values for pNPP and CASP of a similar magnitude.

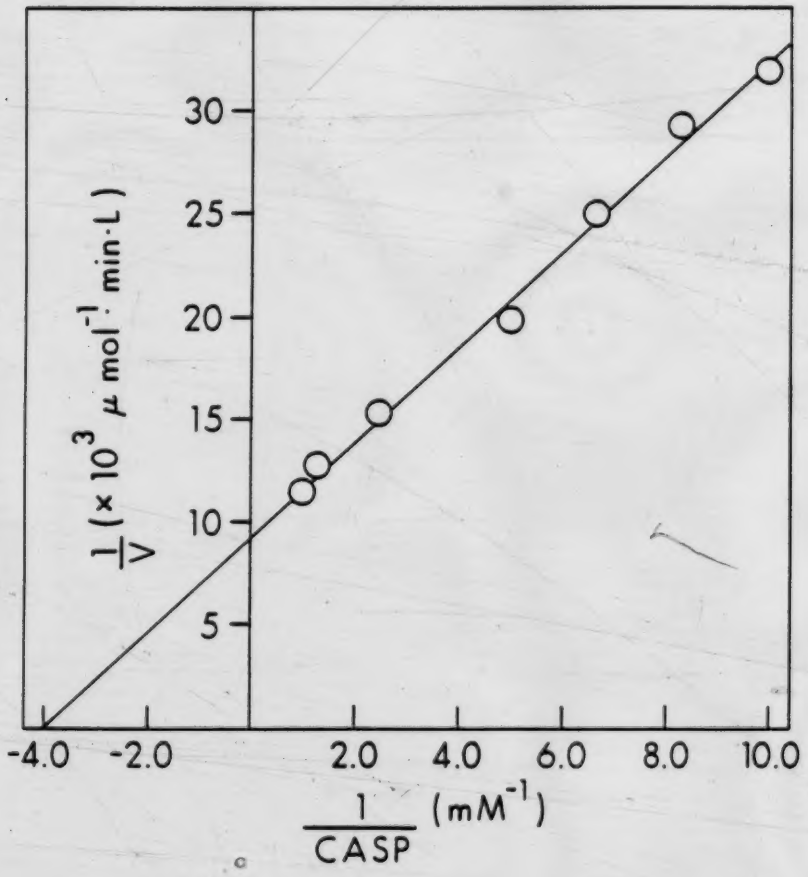


Figure 2. Determination of a Michaelis constant for alkaline phosphatase from human liver utilizing CASP as substrate

Double reciprocal plot obtained from alkaline phosphatase purified from human liver. Hydrolysis of CASP was measured at 412 nm through use of DTNB in 0.78 M AMP buffer, pH 9.0, which contained 1.5 mM $MgCl_2$ as outlined in the Methods section of this chapter. Similar plots were obtained for all other multiple forms of human alkaline phosphatase tested.

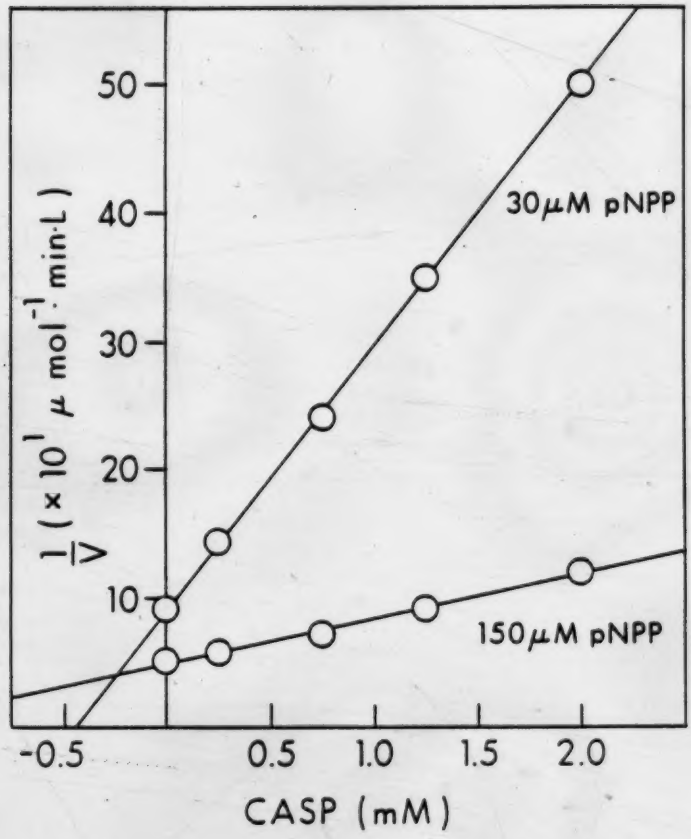


Figure 3. Determination of an apparent K_i for CASP in a mixed substrate system for alkaline phosphatase from human small-intestinal mucosa

Dixon plot obtained for alkaline phosphatase purified from small-intestinal mucosa. Inhibition of pNPP hydrolysis at a concentration of 30 µM and 150 µM was measured over a range of CASP concentrations at 404 nm in 0.78 M AMP buffer, pH 9.0, which contained 1.5 mM $MgCl_2$ as outlined in the Methods section of this chapter. The pattern obtained above is indicative of competitive inhibition and was also obtained for all other multiple forms of human alkaline phosphatase tested.

Table I Enzymic hydrolysis of pNPP and CASP by alkaline phosphatase[a]

Tissue source of enzyme	K_m (mM)[b] for pNPP	K_m (mM)[c] for CASP	Apparent K_i (mM)[d]
Small-intestinal mucosa	0.036	1.00	0.25
Liver	0.019	0.24	0.15
Kidney	0.023	0.44	0.15
Neutrophil	0.032	0.35	0.20
Placenta	0.017	0.09	0.03
Calf Intestine	0.26	0.47	0.24

a All measurements were carried out at 30°C in 0.78 M AMP buffer, pH 9.0, which contained 1.5 mM $MgCl_2$.

b K_m values were determined from double reciprocal plots similar to that shown in Figure 1.

c K_m values were determined from double reciprocal plots similar to that shown in Figure 2.

d Apparent K_i values were obtained from Dixon plots of mixed-substrate experiments in which the inhibition of pNPP hydrolysis was measured, as illustrated in Figure 3.

Values of V_{max} for the multiple forms of alkaline phosphatase could not be determined since the specific activity of each of the purified tissue sources of enzyme was not known due to the difficulty of protein determinations in the presence of Triton X-100. However, relative V_{max} values could be determined under conditions of excess substrate. The ratios of the velocity of pNPP hydrolysis to the velocity of CASP hydrolysis for each multiple form of human alkaline phosphatase using a concentration of 10.0 mM pNPP and 5.0 mM CASP were as follows: small-intestinal mucosa = 1.5, placenta = 1.6, kidney = 1.8, liver = 1.9, and neutrophil = 1.8. In light of the fact that the V_{max} values for the multiple forms of pNPP are quite similar (Stinson and Seargeant, 1981) the similarity amongst the above velocity ratios indicates that the various multiple forms are capable of hydrolyzing CASP at similar rates, although their affinities for CASP are different.

D. DISCUSSION

It is evident from these studies that all of the multiple forms of alkaline phosphatase tested are capable of utilizing CASP as a substrate. It is also apparent that the affinity for CASP amongst the forms is quite variable. In addition, in all the multiple forms of alkaline phosphatase from human tissue sources, the enzymes' affinity for pNPP is much greater than that for CASP.

It is interesting to note that the K_m values for the human multiple forms of alkaline phosphatase can be divided into three groups: a low value for the placental form, an intermediate cluster of values for the liver, kidney and neutrophil forms, and a high value for the

small-intestinal form of the enzyme. This finding is consistent with the proposal that there are three structural genes coding for human alkaline phosphatase. That is, there are three true isoenzymes; the placental type, the intestinal type, and the liver/kidney/bone type. It is of interest to note that neutrophil alkaline phosphatase would also appear to belong to the latter group. This point will be investigated further in Chapter Five.

On a more practical level, the differences in K_m values for CASP amongst the human multiple forms of alkaline phosphatase could be of importance when considering detection of CASP hydrolysis in human serum. Considering that serum normally contains a combination of the multiple forms of alkaline phosphatase, the proportions of which may vary in particular disease states, assay conditions must be employed which will be suitable for detection of all forms of the enzyme. This point will be elaborated on in the following chapter.

The K_m values of CASP and pNPP obtained from calf intestine which are shown in Table I would seem to support the earlier findings of Neumann (1968) when using alkaline phosphatase from E. coli and chicken intestine and perhaps are typical of enzyme obtained from certain animal tissue sources. The findings when using human multiple forms of the enzyme are strikingly different: in all tests involving human alkaline phosphatase the enzymes' affinity for pNPP is much greater than that for CASP. The difference in substrate affinity amongst the human multiple forms is understandable knowing that differences in both structure and properties have been previously

reported from evidence obtained in subunit molecular weight studies, peptide mapping, heat inactivation and chemical inhibition studies (Stinson and Seargeant, 1981).

The data obtained from the Dixon plots reveal that CASP is a competitive inhibitor with regard to pNPP hydrolysis by alkaline phosphatase. This has been previously reported by Neumann et al (1967) and Dulis and Wilson (1978). The values reported by the latter group also demonstrated a range of K_i values determined from different sera. This may be a reflection of the presence of, or varying proportions of, different multiple forms of alkaline phosphatase within each serum.

Inasmuch as CASP is acting as an alternate substrate, rather than strictly as a competitive inhibitor, a true K_i is not determined by these experiments. The normal mechanism of a competitive inhibitor is as follows:

$$\begin{array}{ccccc} E + S & \underset{k_2}{\overset{k_1}{\rightleftharpoons}} & ES & \xrightarrow{k_3} & E + P \\ \Updownarrow & & & & \\ E + I & \underset{k_6}{\overset{k_5}{\rightleftharpoons}} & EI & & \end{array}$$

where K_i is equal to the dissociation constant of the EI complex or $\frac{k_6}{k_5}$. If, as in the case of CASP which we are considering to be I, the EI complex can also dissociate into free E and a second product, the actual dissociation constant must have a second term added to its numerator. However, since the K_m and apparent K_i values obtained are relatively close, and there is a considerable amount of error associated with the DTNB assay of CASP hydrolysis, it is probably safe to assume that the apparent K_i values are indicative of

the true K_i values. This point gains importance when considering a mixed substrate assay in order to measure the amount of CASP hydrolysis in a system. This possibility is examined in the following chapter.

CHAPTER FOUR

A Search for N-Alkaline Phosphatase

A. INTRODUCTION

1. General

As shown in the previous chapter, alkaline phosphatases obtained from various tissue sources are all capable of hydrolyzing cysteamine-S-phosphate (CASP). A number of earlier studies of this type of activity performed by Neumann and co-workers are summarized below since they give rise to several important questions.

In 1970, Neumann et al reported that when either C57BL/6 or AKR mice developed leukemia, there was a substantial rise in tissue alkaline phosphatase levels. In addition, although alkaline phosphatases from Eschericia coli and chicken intestine were previously shown to hydrolyze pNPP and CASP at a similar rate (Neumann et al, 1967; Neumann, 1968), when tissue homogenates of leukemic C57BL/6 mice were studied the hydrolysis of pNPP was greatly enhanced over that of CASP and tissue homogenates from leukemic AKR mice were not capable of hydrolyzing CASP at all. A further study by Neumann et al (1971) reported that in leukemic tissue homogenates from C57BL/6, SJL/J and AKR strains of mice the ratio of the rate of hydrolysis of pNPP to the rate of hydrolysis of CASP was greater than the corresponding ratio using nonleukemic tissue homogenates. In addition, the pattern obtained upon polyacrylamide gel electrophoresis of thymic alkaline phosphatase from leukemic AKR mice contained a unique band. In 1971, Wilson et al reported that when thymic lymphomas were induced by virus in C57BL/6 mice, an elevation

of alkaline phosphatase activity appeared first in the thymus, and then in the other organs, concurrently with dissemination. A study by Haran-Ghera et al in 1972 reported that alkaline phosphatase levels in the thymus and lymph nodes were normal in SJL/J mice with either a reticulum cell neoplasm or a myeloid leukemia, but were elevated in SJL/J mice with lymphatic leukemia.

The relevance of these previous findings with regard to humans was investigated in a study by Neumann et al in 1974. She reported a unique alkaline phosphatase in the sera of patients with lymphatic leukemia and infectious mononucleosis which was distinct from the normal alkaline phosphatase. The enzyme was designated N-alkaline phosphatase; N was derived from the Hebrew word ne-elam meaning unknown. Its distinguishing characteristics were twofold; firstly, the formation of a unique band upon polyacrylamide gel electrophoresis of the serum, and secondly, the lack of hydrolysis of S-substituted monoesters of thiophosphoric acid. Further studies from Neumann's laboratory (Klein et al, 1976; Neumann et al, 1976; Karpas et al, 1978) culminated in a report by Neumann et al in 1979 which claimed that the presence of N-alkaline phosphatase could be used as a potential disease marker for lymphoproliferative disorders since it had been detected in significant amounts in the sera, and in some cases the leukocytes, of most patients with these kinds of disorders, but it was not found in patients suffering from myeloid leukemias, various types of carcinoma, autoimmune disorders, or liver disease.

The existence of an unique enzyme marker for lymphoproliferative disorders would be important both as a diagnostic tool and to follow the course of treatment. However, studies performed by two other

independent groups have been unable to substantiate or confirm the above findings. Dulis and Wilson (1978) compared three normal sera with twenty abnormal sera from patients suffering from infectious mononucleosis, chronic or acute lymphatic leukemia, Hodgkin's disease, chronic or acute granulocytic leukemia, malignant melanoma, or lymphosarcoma. They found that CASP hydrolysis was impossible to measure in serum using the DTNB procedure as outlined by Neumann due to increases in optical density arising from other sources. Furthermore, they were unable to detect a unique band upon polyacrylamide gel electrophoresis of the abnormal sera. A study by Kelly et al (1979) of sera from normal subjects and patients with chronic lymphatic leukemia revealed no significant differences between the two groups in terms of the rate of hydrolysis of pNPP and CASP. In addition, they concluded that the previously reported extraction and purification procedures for N-alkaline phosphatase (Neumann et al, 1976) were unsuitable for human alkaline phosphatases.

In light of the above conflicting reports, a further search for N-alkaline phosphatase seemed to be in order.

2. Quantification of N-alkaline phosphatase

The quantification of N-alkaline phosphatase in serum poses a problem in that it is an absence of activity which one must measure. Therefore, Neumann devised the following system for quantification (Neumann et al, 1979).

Initially she determined the ratio of velocity of hydrolysis of pNPP to the velocity of hydrolysis of CASP using sera from 20 healthy individuals. The ratios obtained ranged from 1.39 - 2.14 with a mean value of 1.63 and a standard deviation of 0.5. Based on this value of

1.6, Neumann then defined the following quantities:

Normal alkaline phosphatase activity = 1.6 (V_{CASP})

N-alkaline phosphatase activity = Total activity - Normal activity

$$= V_{pNPP} - 1.6\ (V_{CASP})$$

$$\% \text{ of N-alkaline phosphatase present} = \frac{\text{N-alkaline phosphatase activity}}{\text{Total alkaline phosphatase activity}} \times 100$$

$$= \frac{V_{pNPP} - 1.6\ (V_{CASP})}{V_{pNPP}} \times 100$$

The ratio values obtained covered such a wide range that the standard deviation was approximately 30% of the mean value. Neumann therefore decided to consider N-alkaline phosphatase as present only when its value represented more than 30% of the total activity.

3. Procedural modifications

Although a detailed outline of the methods used is included in the Methods section of this chapter, a few points are worthy of mention.

Inasmuch as controversy exists as to the actual existence of N-alkaline phosphatase, it would seem logical to conduct a search for it under optimal conditions. It is a well-accepted fact that enzyme activity should be measured under conditions where zero order kinetics are maintained, that is, where the velocity of the reaction is maximal and dependent on only the amount of enzyme present. It follows, therefore, that the velocity should be independent of substrate concentration and for this to be the case, the substrate concentration employed should be at least twenty times the K_m value of the enzyme for that substrate. The K_m values for the multiple enzyme forms which may appear in human serum were presented in Chapter Three, and ranged

from a value of 0.09 mM to 1.00 mM when using CASP as a substrate. Therefore a concentration of 5.0 mM CASP, which was five to fifty times the K_m values, was employed in all assays of CASP hydrolysis with the exception of one set where 1.0 mM CASP was used as suggested by Neumann for purposes of comparison. A higher concentration of CASP could not be employed due to the overwhelming amount of spontaneous CASP hydrolysis and thus DTNB hydrolysis.

Due to this limitation of the DTNB method a mixed substrate method of assay was considered. In this case CASP hydrolysis would be ascertained by the decrease in pNPP hydrolysis due to competition for the enzyme by CASP. This proposal was abandoned for two main reasons. Firstly, it is generally more desirable to make a direct rather than indirect measurement. Specifically, there would be less error involved in measuring a small increase in activity than in measuring a small decrease in a large amount of activity. In addition, as discussed in Chapter Three, the K_i values determined for the enzymes were only apparent. The actual significance and therefore the associated error of the reaction of E-CASP to free enzyme and cysteamine, as opposed to the main reaction of E-pNPP to free enzyme and p-nitrophenolate, is unknown. In light of these two sources of error it seemed more desirable to pursue an approach of direct measurement of CASP hydrolysis. Secondly, whereas in normal serum the rate of hydrolysis of pNPP should decrease upon addition of CASP due to competition for the enzyme, in a serum containing only N-alkaline phosphatase the rate of hydrolysis of pNPP should, according to Neumann, remain the same upon addition of CASP since by her definition the enzyme is incapable of utilizing CASP. However, according to Dulis and Wilson

(1978), a further defining characteristic of N-alkaline phosphatase is that when hydrolyzing pNPP it is very readily inhibited by CASP, 100 to 1000 times more so than is the normal enzyme. Therefore, the rate of hydrolysis of pNPP of a serum containing N-alkaline phosphatase would decrease upon the addition of CASP.

Thus, it would be extremely difficult to distinguish between the presence of normal and N-alkaline phosphatase since both would exhibit a decreased rate of pNPP hydrolysis when CASP was present.

4. Outline of this investigation

Initially the rates of hydrolysis of pNPP and CASP by all of the previously used multiple forms of alkaline phosphatase were determined using both 1.0 mM and 5.0 mM CASP in order to ascertain whether there is in fact a difference between the velocities obtained in each situation, and whether 5.0 mM CASP is closer to representing optimal conditions.

A study of normal sera followed in an attempt to confirm Neumann's reported value of 1.6 for the ratio of the velocity of pNPP hydrolysis to the velocity of CASP hydrolysis by normal human alkaline phosphatases (Neumann et al, 1979).

A study of sera from selected hospitalized patients with three general types of disorder was also carried out. Group I consists of disorders which have been claimed by Neumann to be associated with the presence of N-alkaline phosphatase in the serum, namely non-Hodgkin's lymphomas, infectious mononucleosis, Burkitt's lymphoma, and acute and chronic lymphatic leukemia. Group II consists of proliferative disorders which according to Neumann et al (1979) are not associated with the presence of N-alkaline phosphatase in the serum, namely acute and

chronic granulocytic leukemia and Hodgkin's disease. Proliferative diseases which have not been previously studied for the presence of N-alkaline phosphatase in serum, namely myeloma, sarcomas and lung cancer, are also included within this group. For purposes of comparison Group III consists of conditions where patients have increased levels of serum alkaline phosphatase due either to pregnancy or some kind of disorder other than a proliferative type. Finally, pNPP and CASP hydrolysis were studied in butanol extracts prepared from both neutrophils and lymphocytes of normal individuals since it is of interest to compare the activity of the two cell lines and of serum.

The criteria used for the diagnosis of each of the above disorders appear in Appendix 1.

B. METHODS

The rate of hydrolysis of CASP and pNPP was measured at 30°C essentially as outlined in Chapter Two. The buffer used was 0.78 M AMP, pH 9.0, which contained 1.5 mM $MgCl_2$. When serum was measured for CASP activity the DTNB and CASP blanks were corrected for volume by addition of heat-inactivated serum equal in volume to that of the serum used in the test system. A concentration of 1.0 mM CASP was used for the initial set of studies but 5.0 mM CASP was employed in all the following cases. A concentration of 10.0 mM pNPP was used in all instances.

In the case of lymphocytes, the alkaline phosphatase activity was so low that the cells from several donors had to be pooled in order to obtain measurable activity. Butanol extractions of the granulocyte

and lymphocyte suspensions were performed as outlined in Chapter Two. All sera and butanol extracts were stored frozen at -20°C, and were thawed immediately prior to assay.

For each specimen the velocity of hydrolysis of pNPP and CASP, as well as the ratio between the two, $\frac{V_{pNPP}}{V_{CASP}}$ was determined.

C. RESULTS

1. The effect of CASP concentration on the velocity of pNPP hydrolysis to CASP hydrolysis ratio

The hydrolysis of 1.0 mM and 5.0 mM CASP by each of the purified human enzyme preparations was measured, and the ratio of $\frac{V_{pNPP}}{V_{CASP}}$ determined. The results obtained appear in Table II.

Table II The effect of CASP concentration on the $\frac{V_{pNPP}}{V_{CASP}}$ ratio[a]

Tissue source of enzyme	$\frac{V_{pNPP}}{V_{CASP}}$ at 1.0 mM CASP	$\frac{V_{pNPP}}{V_{CASP}}$ at 5.0 mM CASP
Small-intestinal mucosa	2.4	1.5
Liver	3.3	1.9
Kidney	3.4	1.8
Placenta	2.0	1.6
Mean $\bar{x}$	2.8	1.7

a Velocity of hydrolysis is expressed as µmol CASP hydrolyzed/min/L of enzyme preparation in 0.78 m AMP, pH 9.0, which contained 1.5 mM $MgCl_2$, at 30°C.

Comparing the hydrolysis of 1.0 mM and 5.0 mM CASP, it is evident that the velocity ratio was decreased in all cases when a concentration

of 5.0 mM CASP was employed. Inasmuch as the concentration of pNPP was constant at 10.0 mM, the lowering of the ratios indicates an increase of CASP hydrolysis when the higher substrate concentration was used. Obviously at a concentration of 1.0 mM CASP the substrate had limiting effects on velocity, and therefore zero order kinetics were not maintained. None of the values were close to the previously reported value of 1.6 (Neumann et al, 1979) at a concentration of 1.0 mM CASP. However, perhaps by coincidence, the mean value of the ratios obtained when 5.0 mM CASP was employed was 1.7.

2. Alkaline phosphatase activity in normal sera

Thirty-six sera were screened for normal levels of alkaline phosphatase activity using a Technicon 12/60. The velocity of hydrolysis of both pNPP and CASP, as well as the ratio between the two, were determined for each. An example of the tracings obtained when a normal serum was measured for hydrolysis of CASP appears in Figure 4. Despite a large initial reaction between serum proteins and DTNB the reaction had reached a steady-state after 30 sec. All the velocity values obtained appear in Table III. Although the values for the velocity of hydrolysis of both substrates vary somewhat, the velocity ratios are quite consistent. A value of 2.1 ± 0.5 was obtained for the mean of the ratios as compared with the previously reported value of 1.6 ± 0.5 (Neumann et al, 1979). The coefficient of variation obtained was 24% which is slightly better than Neumann's value of 30% (Neumann et al, 1979). A study by Kelly et al (1979) also reported a value of 2.1 for the velocity ratio between pNPP and CASP in normal sera.

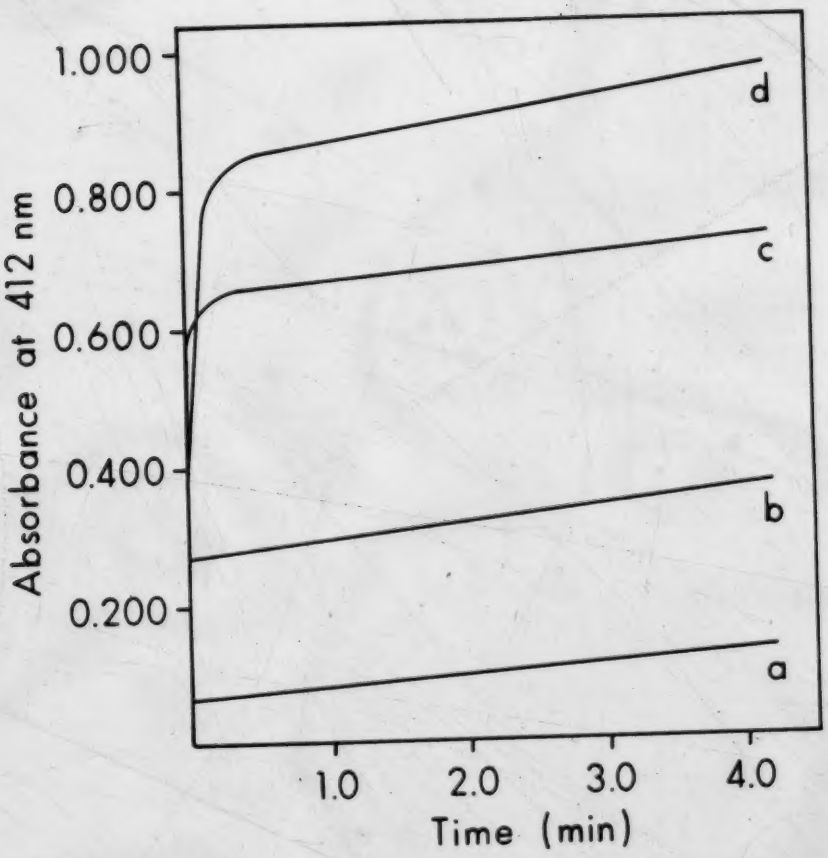


Figure 4. Example assay of CASP hydrolysis in serum

All measurements were performed at 30°C in 0.78 M AMP buffer, pH 9.0, which contained 1.5 mM $MgCl_2$. Cuvettes contained: a, DTNB in buffer; b, DTNB & CASP in buffer; c, DTNB & serum in buffer; d, DTNB & CASP & serum in buffer. The rate of hydrolysis of CASP by alkaline phosphatase in the serum was obtained from the rate of change in absorbance of d - c - (b-a).

Table III Alkaline phosphatase activity in normal sera[a]

Serum number	V_{pNPP}	V_{CASP}	$\frac{V_{pNPP}}{V_{CASP}}$
1	18.6	7.7	2.4
2	14.0	6.5	2.2
3	14.1	8.5	1.7
4	40.0	17.7	2.3
5	20.5	10.9	1.9
6	49.2	26.3	1.9
7	20.2	10.3	2.0
8	23.4	11.9	2.0
9	18.7	6.7	2.8
10	16.4	5.9	2.8
11	22.5	10.3	2.2
12	22.5	8.3	2.7
13	13.3	6.1	2.2
14	11.7	8.4	1.4
15	19.0	6.9	2.8
16	16.5	9.7	1.7
17	18.4	10.5	1.8
18	14.1	8.7	1.6
19	12.6	8.0	1.6
20	12.9	8.1	1.6
21	18.0	9.1	2.0
22	11.9	10.3	1.2
23	13.8	8.3	1.7
24	20.5	11.5	1.8
25	10.5	3.3	3.2
26	15.5	5.6	2.8
27	15.9	7.4	2.1
28	20.6	11.8	1.7
29	17.1	5.7	3.0
30	22.1	8.7	2.5
31	26.2	11.2	2.3
32	13.8	8.0	1.7
33	28.0	12.9	2.2
34	13.8	8.0	1.3
35	24.1	10.7	2.3
36	15.5	10.2	1.5
TOTAL	$\bar{x}$ ± S.D.		2.1 ± 0.5
	c.v.		24%

a Alkaline phosphatase activity is expressed as µmol pNPP or CASP hydrolyzed/min/L of serum in 0.78 M AMP, pH 9.0, which contained 1.5 mM $MgCl_2$, at 30°C.

3. Alkaline phosphatase activity in sera of patients in Group I

The results obtained from sera of patients with disorders of Group I are summarized in Table IV. These results are similar to those obtained with normal serum (Table III) in that although considerable variation within each type of disorder occurs among the individual values for V_{pNPP} and for V_{CASP} the velocity ratios are again quite consistent. When the mean velocity ratio for each of the four types of disorder is calculated and these are compared, a very close similarity amongst them is apparent. More importantly these mean velocity ratios do not differ significantly from those of the normal group shown in Table III. If N-alkaline phosphatase were present in these Group I sera, as claimed by Neumann et al (1979), the velocity ratios would have been greatly increased as compared with those of normal sera since CASP hydrolysis would not have occurred.

4. Alkaline phosphatase activity in sera of patients in Group II

Patients who were suffering from disorders included in Group II also had their sera studied for alkaline phosphatase activity. The results appear in Table V. Once again, the ratio values obtained are very similar to those of the normal group. Although patients with myeloma have a proliferation of B lymphocytes and should therefore be included in the lymphoproliferative group, sera from these patients had not specifically been claimed by Neumann et al (1979) to contain N-alkaline phosphatase. They were therefore placed in Group II.

5. Alkaline phosphatase activity in the sera of patients in Group III

There was the possibility that sera with increased levels of alkaline phosphatase may behave differently from sera with normal levels. For this reason sera from six pregnant women were included since they

Table IV Alkaline phosphatase activity in sera from patients in Group I [a,b]

Type of disorder	V_{pNPP}	V_{CASP}	$\frac{V_{pNPP}}{V_{CASP}}$
Lymphoma	54.6	27.1	2.0
	19.6	11.2	1.8
	13.6	9.6	1.4
	23.2	10.1	2.3
	24.6	10.4	2.4
	29.9	16.8	1.8
	15.2	6.5	2.3
	15.8	7.9	2.0
	13.9	8.7	1.6
	21.9	9.4	2.3
	15.9	7.2	2.2
	18.1	7.6	2.4
	13.5	6.0	2.3
	18.6	7.7	2.4
	19.4	9.6	2.0
	22.3	9.3	2.4
$\bar{x} \pm$ S.D.			2.1 ± 0.3
Infectious mononucleosis[c]	34.0	20.5	1.7
	28.1	14.2	2.0
	6.1	3.1	2.0
	45.1	20.8	2.2
$\bar{x}$			2.0
Chronic lymphatic leukemia[c]	13.9	6.8	2.0
	16.1	5.8	2.8
	13.8	8.2	1.7
$\bar{x}$			2.2
Burkitt's lymphoma[c]	17.2	9.1	1.9
Acute lymphatic leukemia[c]	13.4	10.2	1.3
TOTAL $\bar{x} \pm$ S.D.			2.0 ± 0.4
c.v.			20%

a Alkaline phosphatase activity is expressed as µmol pNPP or CASP hydrolyzed/min/L of serum in 0.78 M AMP, pH 9.0, which contained 1.5 mM $MgCl_2$, at 30°C.

b Group I consists of disorders which have been claimed by Neumann to be associated with the presence of N-alkaline phosphatase in the serum (Neumann et al, 1979).

c Where fewer than five values were available, a S.D. was not calculated.

Table V Alkaline phosphatase activity in sera from patients in Group II[a,b]

Type of disorder	V_{pNPP}	V_{CASP}	$\frac{V_{pNPP}}{V_{CASP}}$
Myeloma	24.3	9.6	2.5
	18.4	10.5	1.8
	16.0	8.5	1.9
	26.3	12.6	2.1
	13.4	3.1	4.3
	18.5	7.1	2.6
	27.0	10.2	2.6
	20.6	7.9	2.6
	22.8	10.5	2.2
	60.3	31.2	1.9
	18.1	7.6	2.4
$\bar{x} \pm$ S.D.			2.4 ± 0.7
Acute myeloid leukemia	13.4	6.9	1.9
	16.3	7.7	2.1
	23.5	11.5	2.0
	23.0	10.2	2.3
	93.3	51.6	1.8
$\bar{x} \pm$ S.D.			2.0 ± 0.2
Lung Cancer	24.6	10.4	2.4
	17.3	7.7	2.2
	13.7	6.1	2.2
	15.0	7.4	2.0
	32.0	14.2	2.3
$\bar{x} \pm$ S.D.			2.2 ± 0.2
Hodgkin's disease[c]	15.8	6.6	2.4
	20.3	9.8	2.1
	76.6	38.4	2.0
	28.1	10.4	2.7
$\bar{x}$			2.3
Sarcoma[c]	11.6	4.7	2.5
	33.8	12.1	2.8
	36.7	20.3	1.8
$\bar{x}$			2.4
TOTAL $\bar{x} \pm$ S.D.			2.3 ± 0.5
c.v.			22%

a Alkaline phosphatase activity is expressed as umol pNPP or CASP hydrolyzed/min/L of serum in 0.78 M AMP, pH 9.0, which contained 1.5 mM $MgCl_2$, at 30°C.

b Group II consists of proliferative disorders which according to Neumann are not associated with the presence of N-alkaline phosphatase in the serum and disorders which have not been previously studied for the presence of N-alkaline phosphatase in the serum (Neumann et al, 1979).

c Where fewer than five values were available a S.D. was not calculated.

are readily available and known to contain high levels of the placental form of alkaline phosphatase. The behaviour of this group of sera is shown in Table VI. The ratio values obtained appear to be slightly lower than those of the normal group (Table III). There is no apparent explanation for this, although the ratio obtained for the purified preparation of placental alkaline phosphatase was lower than that of the liver or kidney forms (Table II).

Table VI Alkaline phosphatase activity in sera from patients in Group III[a,b]

Description of patient	V_{pNPP}	V_{CASP}	$\frac{V_{pNPP}}{V_{CASP}}$
Pregnant	21.0	17.8	1.2
	13.8	8.3	1.7
	53.9	33.0	1.6
	11.1	5.5	2.0
	11.6	7.9	1.5
	26.1	13.4	1.9
$\bar{x}$ ± S.D.			1.7 ± 0.3
Increased enzyme activity[c]	77.5	37.6	2.1
	117.4	53.8	2.2
	104.9	50.7	2.1
$\bar{x}$			2.1
TOTAL $\bar{x}$ ± S.D.			1.8 ± 0.3
c.v.			17%

a Alkaline phosphatase activity is expressed as µmol pNPP or CASP hydrolyzed/min/L of serum in 0.78 M AMP, pH 9.0, which contained 1.5 mM $MgCl_2$, at 30°C.

b Group III includes conditions where patients have increased levels of serum alkaline phosphatase.

c Where fewer than five values were available, a S.D. was not calculated.

Three patients with disorders other than a proliferative type who were known from previous testing to have increased serum levels of alkaline phosphatase were also studied. Results also appear in Table VI. Although the velocity of hydrolysis of each type of substrate was considerably increased, the velocity ratio of pNPP to CASP was still found to be similar to the normal group (Table III).

6. Alkaline phosphatase activity in butanol extracts prepared from normal leukocyte suspensions

Butanol extracts of normal granulocyte and pooled lymphocyte cell suspensions were studied for alkaline phosphatase activity towards pNPP and CASP. The results obtained appear in Table VII. There is no significant difference between the mean velocity ratios obtained from each type of cell; and in both cases the values are similar to those obtained for normal serum. There is no apparent reason for the large coefficients of variation in this group. These differences are perhaps related to the isolation and extraction procedures which the cells have gone through. In the case of the lymphocytes it is difficult to establish whether true lymphocyte activity is observed or whether it is just a reflection of the activity of contaminating neutrophils.

Table VII Alkaline phosphatase activity in butanol extracts prepared from normal leukocyte suspensions[a]

Type of cell	V_{pNPP}	V_{CASP}	$\frac{V_{pNPP}}{V_{CASP}}$
Granulocytes[b]	20.1	6.8	3.0
	25.6	8.3	3.1
	81.6	17.5	4.7
	18.9	10.5	1.8
	7.4	7.6	1.0
	65.8	35.7	1.8
	48.5	26.6	1.8
	39.5	19.8	2.0
	11.1	7.6	1.5
	91.8	46.3	2.0
	43.3	23.9	1.8
	6.7	6.0	1.1
$\bar{x}$ ± S.D.			2.1 ± 1.0
Lymphocytes[b,c]	11.3	7.6	1.5
	10.4	6.3	1.7
	5.2	3.0	1.7
	4.1	2.2	1.9
	6.4	3.9	1.6
$\bar{x}$ ± S.D.			1.7 ± 0.2
TOTAL ± S.D.			2.0 ± 0.9
c.v.			45%

a Alkaline phosphatase activity is expressed as µmol pNPP or CASP hydrolyzed/min/L of cell suspension in 0.78 M AMP, pH 9.0, which contained 1.5 mM $MgCl_2$, at 30°C.

b The number of leukocytes/ml of suspension varied depending on leukocyte yield from whole blood.

c Lymphocytes from several donors had been pooled in order to obtain a measurable amount of enzyme activity.

7. Summary of results

A summary of the mean velocity ratios obtained in the above groups of samples is contained in Table VIII.

Table VIII Summary of the ratios of pNPP to CASP hydrolysis in human sera and butanol extracts of human leukocyte suspensions[a]

Type of sample	$\frac{V_{pNPP}}{V_{CASP}}$	S.D.	c.v.
Normal sera	2.1	± 0.5	24%
Group I sera	2.0	± 0.4	20%
Group II sera	2.3	± 0.5	22%
Group III sera	1.8	± 0.3	17%
Butanol extracts of leukocytes	2.0	± 0.9	45%
TOTAL $\bar{x}$	2.1	± 0.5	24%

a Alkaline phosphatase activity is expressed as µmol pNPP or CASP hydrolyzed/min/L of serum or cell suspension in 0.78 M AMP, pH 9.0, which contained 1.5 mM $MgCl_2$, at 30°C.

The velocity ratios obtained for the five groups studied are all very similar and no significantly large differences in ratio are evident. The average value obtained for the $\frac{V_{pNPP}}{V_{CASP}}$ ratio was 2.1. When Neumann's definition is followed and a value of 2.1 is used for the calculation of the amount of N-alkaline phosphatase activity present in the variety of sera tested the result is below 30% in all cases; in most instances it is well below 30% or zero. Therefore, there appears to be no evidence for the existence of an unique form of alkaline phosphatase either in those disorders in which it had been claimed previously to be present (Neumann et al, 1979) or in a variety of other disorders, both proliferative and non-proliferative.

D. DISCUSSION

The results obtained in the present study support the reports of Dulis and Wilson (1978) and Kelly et al (1979) which challenged the claims of existence of such an enzyme as N-alkaline phosphatase. What then, is the cause of the abnormal activity which Neumann continually seems to find? It is most likely that the answer lies within her assay methods.

There are several aspects of the method used for detection of CASP hydrolysis as outlined by Neumann et al (1979) that make it either unsuitable or at least non-optimal for use with serum. The foremost problem in the original assay method is the concentration of substrates employed. As mentioned earlier a concentration of 1.0 mM CASP is unsuitable for a serum assay since it is known that the multiple forms of alkaline phosphatase which may be present in serum have K_m values for CASP ranging from 0.09 mM to 1.00 mM (Chapter Three, Table I). Further, in this study the velocity ratios of all of the multiple forms of human alkaline phosphatase increased when the CASP concentration was increased from 1.0 mM to 5.0 mM (Table II). Obviously a lack of substrate was limiting the rate of reaction when a concentration of 1.0 mM CASP was employed. Theoretically for assays with 1.0 mM CASP the greatest error would occur when the intestinal form of alkaline phosphatase was present since its K_m value and the substrate concentration would be the same. It is now known that many individuals who are blood group O or B, secretors, or Lewis-b positive (approximately 60% of the population) possess an intestinal component in their total serum alkaline phosphatase. Therefore an assay method

for human serum which employs a concentration of only 1.0 mM CASP is not acceptable. In this study a concentration of 5.0 mM CASP was employed for all sera and cellular extract assays of CASP hydrolysis.

The highest K_m value for pNPP obtained from the multiple forms of human alkaline phosphatase was 0.03 mM. Therefore, a concentration of 1.0 mM pNPP should be more than adequate in order to overcome any substrate inhibition. However, as Dulis and Wilson (1978) pointed out, there is still a significant problem concerning competition by phosphate esters contained within the serum for the enzyme. These authors found that when a six-fold dilution of normal serum was used with 1.0 mM pNPP more than twice as much phosphate was produced as p-nitrophenol. This was due to serum phosphate esters which acted as substrate in addition to pNPP. However, this type of interference can be substantially reduced through use of 10.0 mM pNPP as is commonly employed in routine alkaline phosphatase assays. Therefore by increasing the substrate concentrations to 5.0 mM for CASP and to 10.0 mM for pNPP both substrate inhibition and serum phosphate ester interference should be virtually eliminated.

A second major flaw in Neumann's assay method of CASP hydrolysis is the absence of a sample blank. Although a blank which contained DTNB, CASP and buffer was included it would account only for the spontaneous hydrolysis of DTNB and CASP. However, the reaction of sulfhydryl groups of serum proteins is extensive although slow and occurs with a declining rate over a period of 30 min (Dulis and Wilson, 1978). This factor gains even more significance when large sample volumes are employed in order to detect low levels of activity. Therefore a sample blank which consisted of DTNB, serum and buffer was

included in all assays in this study (Chapter Two).

Due to the fact that measurement of a low amount of activity is required it is desirable to maximize activity levels by using conditions which are optimal. All of Neumann's work was performed at 22° or 23°C, as was that of Dulis and Wilson (1978), while Kelly et al (1979) employed 25°C. By contrast, a value of 30°C was employed in this study.

The lack of magnesium ions used in Neumann's assay system constitutes another basis for criticism. The stimulating effects of magnesium ions on many alkaline phosphatases, especially those obtained from mammalian tissue sources, are well known. It is generally recommended that magnesium be included in all such assay systems although there is some controversy as to whether additional magnesium is really necessary in the case of serum determinations since the specimen itself contains almost optimal amounts of the cation (McComb et al, 1979). However, in this case where the measurements are in the lower range of sensitivity and the sera were obtained from individuals with various disorders, it seemed more desirable to be confident of the presence of an adequate concentration of cation.

Although all of the previously mentioned authors performed their assays in TRIS/HCl, in this study AMP buffer was chosen because of its widespread use in studies of mammalian tissue alkaline phosphatases. While TRIS/HCl, with a pK_a of 8.0, is an excellent buffer for *E. coli* alkaline phosphatase which has a pH optimum near pH 8.0, it has poor buffering capacity at pH 10.0 and is therefore unsuitable for measuring alkaline phosphatase from mammalian tissue sources.

The pK_a of AMP is 9.3 so it was suitable for use at pH 9.0 where all of the assays in this part of the study were performed.

Another major complaint about Neumann's assay, as pointed out by Dulis and Wilson (1978), is the length of time required for each measurement. Several problems can be encountered when measurements are taken over a period of 20 - 60 min. Neumann et al (1979) indicated that reoxidation by air of cysteamine may occur but reoxidation of 5-thio-2-nitrobenzoic acid can also be a problem. As mentioned earlier, Dulis and Wilson (1978) found that the reaction of DTNB with thiol groups present in the serum was significant over a period of 30 min. This resulted in nonlinear kinetics of both the DTNB-serum control and the test system which made the assignment of an overall value for the total system very difficult. In the present study, when all of the outlined changes in method had been made, the reaction was fast enough that a maximum of 4 min was required for each measurement. Under these new conditions there was a large initial reaction between serum proteins and DTNB but after 30 sec the reaction reached a steady state (Figure 4). Due to the short period of assay reoxidation of either DTNB or CASP was not a problem. In addition no more than 50 µL of sample was ever required.

Although vast improvements were obtained with the outlined modifications in procedure, there was still an inherent problem of considerable magnitude. There is always a certain measure of inaccuracy and insensitivity associated with a system in which the values of the blanks constitute a major part of the total value. It was for this reason that alternative assay methods for CASP hydrolysis were sought. Unfortunately, the mixed substrate system considered in the

present study, plus attempts to utilize ^{32}P-labelled CASP (Kelly et al, 1979) were unsuccessful and nothing more appropriate has yet been discovered.

The average ratio of the velocity of pNPP hydrolysis to the velocity of CASP hydrolysis in human serum was 2.1 in the present study (Table III). The discrepancy between this value and Neumann's value of 1.6 (Neumann et al, 1979) is presumably due to the differences in assay conditions.

Sera from patients with Group I disorders should, according to Neumann et al (1979), contain N-alkaline phosphatase. If N-alkaline phosphatase were indeed present in these sera very low or absent CASP hydrolysis and thus very high velocity ratios should have been obtained in Group I. This was not found to be the case (Table IV); the mean velocity ratio was very similar to that of the normal group (Table III). The sera in Group II were from patients with disorders which Neumann et al (1979) had claimed did not contain N-alkaline phosphatase (acute myeloid leukemia and Hodgkin's disease) or with proliferative disorders which Neumann et al did not examine for the presence of N-alkaline phosphatase (myeloma, lung cancer, and sarcoma). The mean velocity ratio of Group II was again very similar to normal (Table III & Table V). Based on Neumann's hypothesis one would expect N-alkaline phosphatase to be present in the sera of myeloma patients since myeloma is a lymphoproliferative disorder. The possibility that a change in velocity ratio may occur when abnormal levels of alkaline phosphatase are present was also investigated. Group III included sera from patients with an increase in the placental form of alkaline phosphatase because of pregnancy and sera with overall increased levels of alkaline phosphatase.

No significant change in the velocity ratio was detected in either case (Table VI).

Neumann et al (1976) also claimed to have isolated and purified N-alkaline phosphatase from a Burkitt lymphoma cell line and a Moloney-virus-induced murine leukemia cell line. Further claims of the presence of N-alkaline phosphatase within cells were made in a recent report by Neumann and co-workers (Flemans et al, 1981). They reported that N-alkaline phosphatase was present in both the serum and the peripheral blood lymphocytes of a patient with malignant lymphoma and furthermore, that a proportion of the patient's large lymphocytes were cytochemically positive for alkaline phosphatase. Unfortunately in the present study leukocytes could not be obtained from the patients with lymphoproliferative disorders but butanol extracts of granulocytes and lymphocytes were prepared from the normal donors. The alkaline phosphatase from granulocytes acted in a similar manner to that in normal serum (Table VII). Since the alkaline phosphatase activity in lymphocytes was extremely low the lymphocytes from several donors had to be pooled in order to obtain measurable activity. The behaviour of these pools was also similar to that of normal sera (Table VII). Thus it would appear that the alkaline phosphatase present within leukocytes has properties similar to the enzyme present in sera.

The other phenomenon which Neumann et al (1976) used as evidence for the existence of N-alkaline phosphatase is its supposedly unique electrophoretic mobility. This aspect of methodology also has associated problems.

Neumann et al (1971) first mentioned the appearance of an unique enzyme band when they compared the electrophoretic patterns obtained on acrylamide gels of thymic alkaline phosphatase from leukemic and non-leukemic AKR mice. The thymic extracts of leukemic AKR mice demonstrated an extra alkaline phosphatase band which had a fast mobility. However, differences in mobilities were also seen between different mice strains and before and after performance of butanol extractions. In other words variable patterns were obtained not only between leukemic and non-leukemic tissue sources but also between various stages of the experimental procedure. Minor methodological differences in procedure have been previously reported to cause profound changes in mobility. For example, artifactual alkaline phosphatase bands or peaks have been reported to be due to molecular aggregates (Smith and Fogg, 1972), to material incompletely separated from membranes (Eguchi et al, 1972) and to material adsorbed onto contaminants (Beratis et al, 1970). It is interesting to note that Neumann's group in their latest study claimed that a patient contained major amounts of N-alkaline phosphatase in both his serum and lymphocytes (Flemans et al, 1981). However, comparable bands in the electrophoretic patterns obtained from the serum and a butanol extract of the lymphocytes could not be found. Upon subsequent treatment of the lymphocyte extract by passage through a gel filtration column containing a detergent a band comparable to that of the N-alkaline phosphatase contained in the serum was obtained. It is very difficult to ascertain the validity of these findings since the samples contained numerous bands and variable R_f values.

Finally, it should be noted that Neumann et al have always used β-naphthyl phosphate and diazonium salt (Fast Blue) to visualize the alkaline phosphatase bands obtained after electrophoresis. In only one case was an elution of the unique band performed and hydrolysis of CASP measured. Since the hydrolysis was negligible the presence of N-alkaline phosphatase was assumed. From then on the assumption has been that if aberrant catalytic activity is found in the serum the responsible enzyme appears as a fast band on electrophoresis. It was Dulis and Wilson (1978) who actually attempted to demonstrate the catalytic properties of N-alkaline phosphatase on the gel. They were searching for a unique enzyme in the sera of patients with lymphoproliferative disorders which would show up with an α-naphthyl phosphate activity stain but not with a CASP activity stain. They could detect no such unique band in the electrophoretic pattern.

In conclusion, in this study no evidence was found of an unique enzyme form in the sera of patients with a variety of proliferative disorders when biochemical measurements of CASP hydrolysis were made under optimal conditions. The fact that Neumann and co-workers seem to have been able to detect aberrant activity may simply be a reflection of the inaccuracies and insensitivity of their methods. The results obtained in the present study, support those of two other independent groups (Dulis and Wilson, 1978; Kelly et al, 1979) and call into question the validity of the whole proposal of the existence of a unique alkaline phosphatase, N-alkaline phosphatase.

CHAPTER FIVE

Granulocyte Alkaline Phosphatase

A. INTRODUCTION

1. Determination of granulocyte alkaline phosphatase activity

Leukocyte alkaline phosphatase has been a subject of study for several decades. Although most of the early work on alkaline phosphatase in granulocytes was performed by biochemical methods of assay, these methods never really became popular as a routine means of enzyme quantitation, presumably because of the lengthy procedures involved.

Conversely the introduction by Gomori (1939) of a cytochemical technique for the detection of granulocyte alkaline phosphatase has resulted in numerous studies, and several modifications and improved procedures have since been developed. The simplicity of this type of method makes it ideal for use as a routine hematological test for the detection of granulocyte alkaline phosphatase. However, the assessment of activity is subjective and semiquantitative at best.

Despite the difficulties, a number of studies have been performed which employed a biochemical assay of leukocyte alkaline phosphatase either to measure the level of enzyme in various medical conditions (Valentine and Beck, 1951; Park, 1970; Findlay and Johnston, 1977) or to determine the correlation between results obtained by a biochemical and a cytochemical method of assay (Wiltshaw and Moloney, 1955; Meislin et al, 1959; Diamant et al, 1971). However it is very

difficult to compare the results obtained by these various workers since they employ different extraction procedures, assay methods and means of expressing the results.

In addition a great problem, encountered not only in the above studies but throughout the literature, is the way in which the terms leukocytes, granulocytes and neutrophils have been used so loosely. In reality each group represents a subset of the former group yet it has been common practice to use these labels interchangeably. The vast majority of alkaline phosphatase in white blood cells occurs in the neutrophils although small amounts have also been detected in lymphocytes and eosinophils (McComb, Bowers and Posen, 1979). Therefore, although mixed cell populations which contained all types of leukocytes were used in all of the above studies, it was assumed that the detected activity was that of the neutrophils. Since lymphocytes normally constitute from 20 to 45% of the white cell population this assumption may not, in fact, be valid. Inasmuch as eosinophils represent only 1 to 6% of the leukocytes normally present activity due to their presence may be discounted.

Since the majority of alkaline phosphatase activity of white cells occurs in the neutrophils, it is obviously more correct to express the enzymic activity in terms of the number of neutrophils present as opposed to the number of leukocytes present. Therefore, in this study, the lymphocytes were removed from the cell population before alkaline phosphatase activity was determined by a biochemical method of assay. However the results are still expressed as enzymic activity per the number of granulocytes present, rather than the number

of neutrophils, since eosinophils and basophils were not removed before assay.

2. Inhibitors of alkaline phosphatase

In 1963, Fishman reported that L-phenylalanine acted as an organ specific and stereo-specific inhibitor of intestinal alkaline phosphatase. Since that time, the use of specific inhibitors has become popular as a means of differentiation between various multiple forms of human alkaline phosphatase.

Patterns obtained upon heat inactivation and urea inhibition have been employed to establish the presence of certain multiple forms of alkaline phosphatase but urea was reported to produce different inhibition patterns depending on the concentration used (Bahr and Wilkinson, 1967). Levamisole was reported to inhibit liver, kidney and bone forms of alkaline phosphatase, but not intestinal and placental forms (Van Belle, 1976). Various amino acids have also been studied as inhibitors of alkaline phosphatase. Fishman and Sie (1970) reported that L-homoarginine strongly inhibited bone and liver alkaline phosphatase, but had no effect on the enzyme from placenta or intestine. In 1977, Doellgast and Fishman studied the inhibition effects of L-leucine and tripeptides which contained L-leucine. They reported that when L-leucylglycylglycine was used, placental alkaline phosphatase was strongly inhibited but that inhibition of the intestinal isoenzyme was weak. This was an important discovery because in all of the above cases the placental and intestinal forms reacted very similarly and it was therefore difficult to differentiate between them. Mulivor, Plotkin and Harris (1978) developed this point further and presented a scheme which allowed easy differentiation of the three

human isoenzymes of alkaline phosphatase (liver/kidney/bone, intestinal and placental) based upon the clear-cut inhibition patterns exhibited by each in the presence of L-phenylalanine, L-homoarginine, L-leucine, L-leucylglycylglycine and L-phenylalanylglycylglycine.

The procedure of Mulivor et al (1978) was employed in this study to determine which of the three patterns granulocyte alkaline phosphatase exhibits.

B. METHODS

A random selection was made from whole blood units which had been freshly drawn at a regular Red Cross blood donor's clinic. No information concerning the age or sex of the donors was available. Each unit contained 63 mL of citrate-phosphate-dextrose and 450 mL ± 10% of whole blood. A 10 mL aliquot from each unit was drawn into a Vacutainer tube which contained heparin. A volume of 20 µL was removed and suspended in 10.0 mL of Isoton. Two drops of Zap-oglobin was added and a white cell count performed on a Coulter Counter Model B. A blood smear was also prepared from each specimen.

The granulocytes and mononuclear cells were isolated from the remainder of each sample as outlined in Chapter Two. The mononuclear cell layers from several samples were removed, pooled and frozen at -20°C until butanol extractions were performed. Each granulocyte pellet was resuspended in 2.5 mL of Isoton and a smear was prepared. A Wright's stain (see Dacie and Lewis, 1975) was performed on both the whole blood and granulocyte pellet smears and the proportions of granulocytes and mononuclear cells were determined microscopically. A further 1/100 dilution was made in 10.0 mL of Isoton and a

white cell count performed as outlined above. The initial granulocyte suspensions were centrifuged for 10 min at 2400 rpm and the pellets resuspended in 1.0 mL of 10.0 mM TRIS/HCl, pH 7.6, which contained 1.0 mM $MgCl_2$ and 0.1 mM $ZnCl_2$. Butanol extractions were performed on the cell suspensions as outlined in Chapter Two, and the extracts were frozen at -20°C until assayed.

All assays for alkaline phosphatase activity were performed in 0.78 M AMP buffer, pH 10.3, which contained 1.5 mM $MgCl_2$, essentially as outlined in Chapter Two. A concentration of 10.0 mM pNPP and a sample volume of 50 µL was employed in all cases. Protein content was quantitated as outlined in Chapter Two.

For the inhibition studies, 20 µL of a stock solution of an appropriate inhibitor was added to 1.0 mL of a 10.0 mM pNPP solution in 0.78 M AMP, pH 10.3, which contained 1.5 mM $MgCl_2$. The inhibitors employed and their resultant concentrations were: 1.0 mM L-phenylalanylglycylglycine (PGG), 2.5 mM L-phenylalanine (Phe) and 10.0 mM L-homoarginine (Hrg). The reaction was initiated by addition of 50 µL of a butanol extract of a granulocyte or lymphocyte suspension. The rate of hydrolysis of pNPP in the presence of the inhibitor was measured spectrophotometrically at 404 nm. The remaining activity in the presence of inhibitor was expressed as a percentage of the original activity in the absence of the inhibitor. Fifteen butanol extracts were chosen in order to include a range of enzymic activity from 9.8 - 34.8 $mU/10^7$ granulocytes. Measurements were repeated at least once with good agreement.

The lymphocyte pools were thawed, had butanol extractions performed on them, and were then used in the same manner as the granulocyte butanol

extracts for inhibition studies.

A similar protocol was employed using crude butanol extracts of human liver, placenta and intestine. Alkaline phosphatase which was purified essentially as outlined by Seargeant and Stinson (1979) from human liver, placenta, intestine and neutrophils was also studied. Measurements with each inhibitor were repeated at least five times in order to determine a range of results for each type of enzyme tested.

C. RESULTS

1. Determination of granulocyte alkaline phosphatase activity

The white blood cell count and the percentage of granulocytes which appeared in the differential cell count were used to calculate the number of granulocytes present in the original 10 mL of whole blood. The mean results of fourteen smears prepared from the suspensions of isolated granulocytes revealed the following composition: granulocytes-90%, mononuclear cells - 2%, erythrocytes - 8%. Therefore, the white cell populations which were counted were assumed to have a granulocyte purity of 98%. In this manner the absolute number of granulocytes which underwent extraction with butanol could be determined. This value, as well as that of the original number of granulocytes present, was then used to calculate the percentage of granulocyte recovery during the isolation procedure. These figures all appear in Table IX. Examination of the data reveals that there is great variability within each set of values. A scatter plot and regression line for the data appear in Figure 5. A coefficient of correlation of -0.12 ($p > 0.1$) was calculated which indicates that

Table IX Recovery of isolated granulocytes

Specimen number	Granulocytes in 10 mL whole blood[a] ($\times 10^7$)	Granulocytes in resuspension of pellet[b] ($\times 10^7$)	Granulocyte recovery (%)
1	3.30	2.14	73
2	3.45	2.27	66
3	3.30	2.05	62
4	3.13	1.65	53
5	4.97	3.03	61
6	3.35	1.93	58
7	5.29	2.65	50
8	2.52	1.70	67
9	4.27	1.99	47
10	4.27	1.76	41
11	3.49	1.83	52
12	4.34	2.96	68
13	2.15	1.45	67
14	2.92	2.53	87
15	2.76	2.39	87
16	2.78	1.79	64
17	3.67	2.39	65
18	4.16	2.55	61
19	3.48	2.33	67
20	2.33	1.17	50
21	4.61	2.73	59
22	2.92	1.62	55
23	3.67	1.65	45
24	4.56	2.98	65
25	3.95	3.15	80
26	2.18	1.16	53
27	3.87	2.23	58
28	3.73	1.30	35
29	5.09	2.88	57
30	4.33	1.99	46
31	4.87	2.57	53
32	2.50	1.48	59
33	5.11	2.53	50
34	4.42	2.63	60
35	4.45	2.14	48
36	2.52	0.98	39
37	1.58	0.90	57
38	1.97	1.76	89
39	2.04	1.53	75
40	2.88	2.23	77
41	3.57	2.68	75
42	2.22	1.90	86
43	2.76	1.32	48
44	3.79	2.82	74
45	2.31	1.65	71
46	1.68	1.21	72
47	2.28	1.42	62
48	2.04	1.40	69
49	4.09	2.96	72
50	2.62	2.00	76
x ± S.D.	3.37±1.00	2.0±0.60	62±13
c.v.	30%	29%	21%

a Based on the white cell count and the % granulocytes which appeared in the differential cell count.

b The absolute number of granulocytes was represented by 98% of the total cell count for the suspended pellet obtained upon Lymphoprep separation due to 2% contamination by lymphocytes.

c Represented by $\frac{\text{Granulocytes in resuspension of pellet}}{\text{Granulocytes in whole blood}} \times 100$

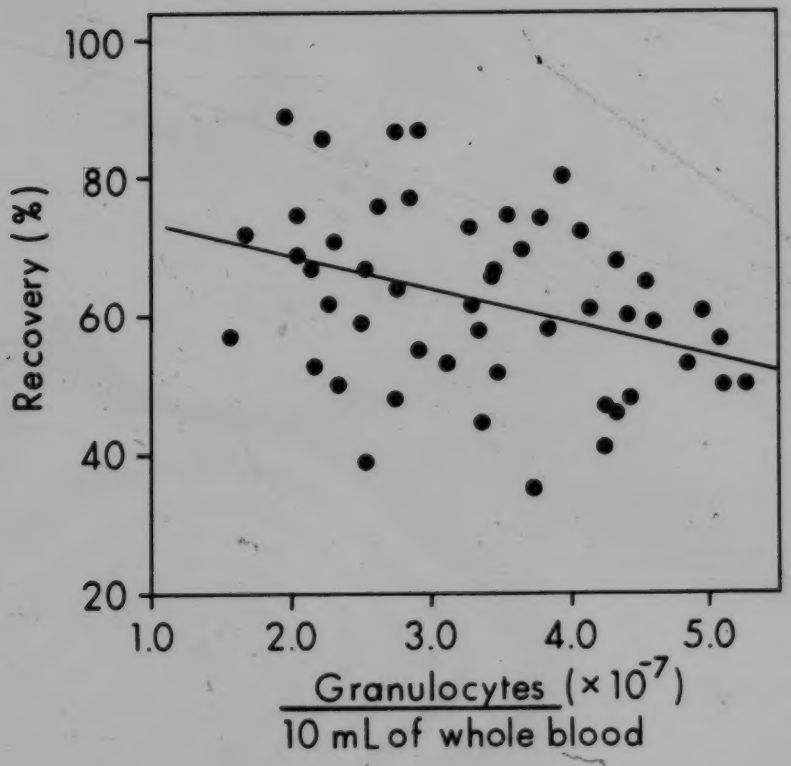


Figure 5. Relationship between the number of granulocytes present in the origihal sample and the percent recovery values

A correlation coefficient of -0.12 ($p > 0.1$) was calculated which indicates that there is no significant correlation between the number of granulocytes present in the original 10 mL of sample and the percentage of granulocytes recovered during the isolation procedure.

there is no significant correlation between the original number of granulocytes present and the percent recovery values.

The alkaline phosphatase activity in each butanol extract, as well as the activity related to the number of granulocytes which underwent extraction, appears in Table X. Again great variability is apparent, this time amongst the values of activity per mL of butanol extract (c.v. = 46%), although this variability is slightly reduced when the activity is expressed per 10^7 granulocytes (c.v. = 36%). The alkaline phosphatase activity in the granulocyte butanol extracts was plotted against the number of granulocytes present. An apparently random distribution was the result, and a correlation coefficient of -0.09 ($p > 0.1$) was obtained. Therefore the level of alkaline phosphatase present in each butanol extract seems to be independent of the number of granulocytes which underwent extraction.

A protein determination was also performed on each butanol extract. Results ranged from 0.5 to 2.5 mg. Since the smears of the granulocyte suspensions revealed erythrocyte contamination of only 8% on a cellular basis, the protein concentrations could be related to the granulocyte counts. The scatter plot and regression line obtained when the number of granulocytes was plotted against the protein concentration appears in Figure 6. The coefficient of correlation is 0.60 ($p < 0.001$). Since the correlation between the two values is highly significant the conversion factor for changing the number of granulocytes to mg protein is represented by the slope of the curve, which is 0.36. Inasmuch as 10^7 granulocytes correspond to 0.36 mg of protein, the enzymic activity expressed per mg of

Table X Alkaline phosphatase activity in normal granulocytes[a]

Specimen number	Activity in butanol extract[b] (U/mL)	Activity per 10^7 granulocytes[c] (mU/10^7 granulocytes)
1*	0.050	20.7
2*	0.032	14.1
3*	0.036	17.6
4*	0.022	13.3
5	0.050	16.5
6	0.025	13.0
7	0.037	14.0
8	0.031	18.2
9	0.031	25.6
10	0.050	28.4
11	0.018	9.8
12*	0.052	17.6
13*	0.024	16.6
14	0.050	19.8
15	0.033	13.8
16*	0.017	9.5
17*	0.039	16.3
18	0.041	16.1
19	0.031	13.3
20	0.020	17.1
21	0.040	14.7
22	0.025	15.4
23	0.042	25.5
24	0.075	25.2
25	0.056	17.8
26	0.023	19.8
27	0.054	24.2
28	0.039	30.0
29*	0.068	23.6
30	0.030	15.1
31	0.060	23.3
32	0.038	25.7
33	0.035	13.8
34	0.055	20.9
35	0.032	15.0
36*	0.020	20.4
37	0.025	27.7
38	0.064	36.4
39*	0.044	28.8
40	0.033	14.8
41*	0.084	31.3
42	0.029	15.3
43*	0.014	10.6
44	0.036	12.8
45	0.017	10.3
46*	0.032	26.4
47	0.047	33.1
48	0.017	12.1
49*	0.100	33.8
50	0.027	13.5
x ± S.D.	0.039±0.018	19.4±6.9
c.v.	46%	36%

a One unit of enzyme activity corresponds to one µmol of substrate hydrolyzed/min at 30°C.
b Butanol extracts were prepared from isolated granulocytes.
c The number of granulocytes present in the pellet which underwent butanol extraction was determined in Table VIII.
* Specimens which were chosen for inhibitor studies (see Figure 7).

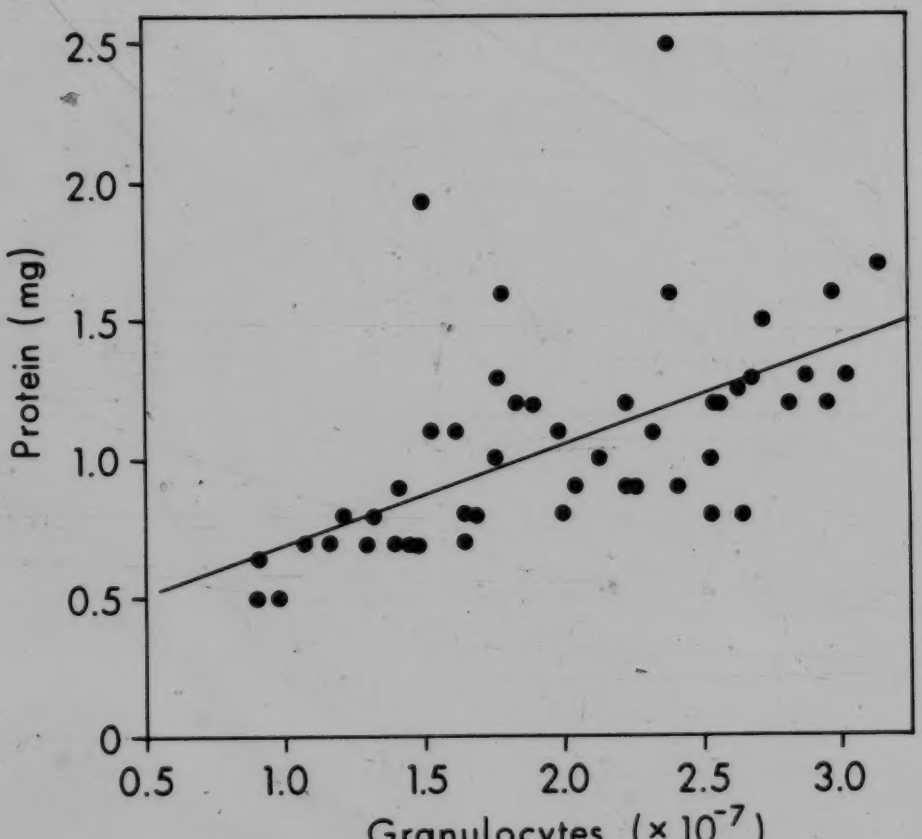


Figure 6. Relationship between the number of granulocytes present and the protein concentration

A correlation coefficient of 0.60 ($p < 0.001$) was calculated which indicates that there is a highly significant correlation between the number of granulocytes which underwent extraction with butanol and the concentration of protein in the aqueous solutions of the butanol extracts.

protein may be obtained by multiplying the values of activity per 10^7 granulocytes by a factor of 2.78. Results were expressed in this study as activity per 10^7 granulocytes since quantitation of activity by cytochemical methods is at a cellular level and it is the level of enzyme per cell which correlates best with disease states (Wilson et al, 1981).

2. Inhibitors of granulocyte alkaline phosphatase

The patterns obtained for each purified alkaline phosphatase isoenzyme when L-phenylalanylglycylglycine (PGG), L-phenylalanine (Phe) and L-homoarginine (Hrg) were used as inhibitors appear in Figure 7. It is evident that each isoenzyme exhibits a characteristic pattern. The patterns obtained with crude butanol tissue extracts were virtually identical to the patterns of their purified tissue counterparts with the exception of the intestinal enzyme. In this case a mixed inhibition pattern was obtained for the crude extract. However, upon heating, the interfering substance was inactivated and an intestinal pattern of inhibition was obtained.

Fifteen crude extracts of granulocytes were chosen as indicated in Table IX. The results obtained when the extracts were tested with the inhibitors also appear in Figure 7. The pattern displayed is practically identical to that of the liver alkaline phosphatase. It is also evident that granulocytes contain a homogeneous alkaline phosphatase population since a mixed pattern of inhibition was not obtained. In addition, there was no evidence that variations in the level of alkaline phosphatase present in the granulocytes are related to variations in the type of isoenzyme present since the same pattern was obtained from extracts which contained a wide range of alkaline

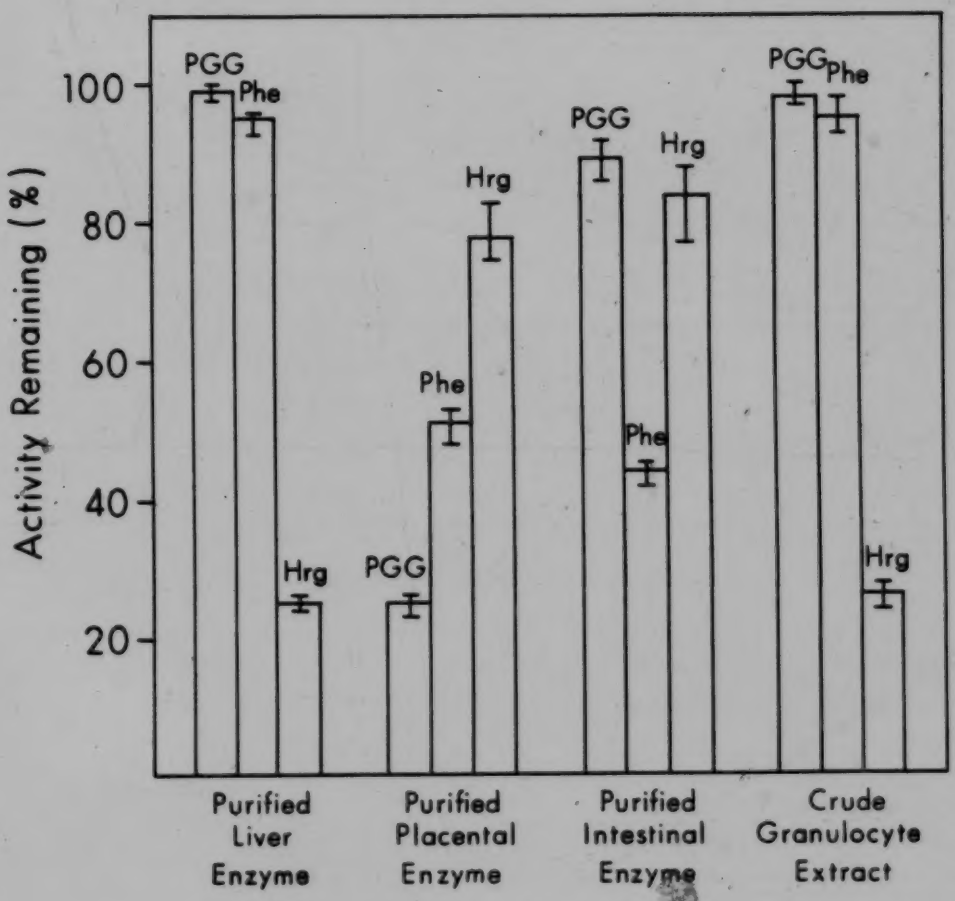


Figure 7. Inhibition patterns of human alkaline phosphatases

Inhibition patterns of alkaline phosphatases using L-phenylalanyl-glycylglycine (PGG), L-phenylalanine (Phe) and L-homoarginine (Hrg) as inhibitors. Results are expressed as the percentage of original activity remaining in the presence of each inhibitor. Bars indicate the range obtained for each purified enzyme repeated five times and the range for duplicate measurements of 15 crude extracts of granulocytes.

phosphatase levels. The pattern obtained from alkaline phosphatase purified from human neutrophils was virtually identical to that of the crude granulocyte extracts.

Butanol extracts of five different lymphocyte pools were also tested with the inhibitors. The mean values and ranges of the percentages of remaining activity were as follows: PGG = 96% (Range = 92 - 99), Phe = 92% (Range = 84 - 98) and Hrg = 32% (Range = 24 - 41). Again this is a pattern similar to that of the liver isoenzyme although it is difficult to evaluate the validity of these results for two reasons. Firstly, the alkaline phosphatase activity in the lymphocyte pools was very low. The activity after inhibition therefore was also extremely low and poor curves were obtained which made measurement difficult. This is probably the explanation for the larger ranges of remaining activity. Secondly, the purity of the lymphocyte population used is of utmost importance since lymphocytes contain only very small amounts of alkaline phosphatase and contaminating granulocytes contain large amounts. Although the lymphocyte populations were estimated microscopically to be 95% pure it is virtually impossible to determine whether the activity which is detected is really that of the lymphocytes or if it is that of contaminating granulocytes. This problem could probably be alleviated by use of a better cell separation technique such as cellular affinity chromatography or cell sorting.

D. DISCUSSION

As previously mentioned the majority of alkaline phosphatase in white blood cells is present within the granulocytes. When a cyto-

chemical method of assay is employed alkaline phosphatase activity is determined only in mature neutrophils and the results are semi-quantitative. When a biochemical method of assay is employed, activity is quantitated. Thus, in the former case the proper population is studied but quantitative results are not obtained and in the latter case, activity is quantitated. However biochemical assays in the past were used on mixed leukocyte populations with variable proportions of each type of white blood cell.

It seems desirable to use a quantitative method since reports have indicated that cellular levels of neutrophil alkaline phosphatase vary in different physiological conditions. For example, Wilson et al (1981) reported a quantitative decrease in alkaline phosphatase per neutrophil in cases of chronic granulocytic leukemia, and an increase in level per cell during pregnancy. Therefore a biochemical method of assay would seem the better choice, but erroneous results could be obtained if this method were used on a general leukocyte population which contained either a high proportion of lymphocytes compared to granulocytes, or lymphocytes with abnormally high activity. The latter possibility has recently been reported by Flemans et al (1980) but has not been confirmed by other investigators. A biochemical assay on a population of granulocytes would thus seem the most desirable method and was therefore the one employed in the present investigation. The addition of a cell separation step did not add significantly to either the length or complexity of the procedure and a granulocyte population of 98% purity was obtained.

If, as is common practice, the reference range is considered to be ± 2 S.D. either side of the mean, then the reference range of alkaline phosphatase activity in granulocytes in this study would be 5.8 - 34.2 mU/10^7 granulocytes. Although this range seems extraordinarily wide similar results have been found in a number of other investigations. Valentine and Beck (1951) reported a range of 13.4 - 50.8 mg of phosphorous liberated/hr by 10^{10} leukocytes. Meislin et al (1959) found a range of 0 - 160 mg of phosphorous liberated/hr by 10^{10} leukocytes. The extent of the range of values detected in the normal population can most likely be explained in terms of the following set of observations. Rosner and Lee (1965) reported a significant difference in leukocyte alkaline phosphatase levels between the sexes: premenopausal women exhibited levels up to 50% greater than men (a mean leukocyte alkaline phosphatase level of 35 as opposed to 23 mg phosphorous liberated/hr by 10^{10} leukocytes) while post-menopausal women and men of 65 years or over displayed similar levels. Additionally, children contained considerably higher levels of the enzyme than adults (a mean level of 68 mg phosphorous liberated/hr by 10^{10} leukocytes), although no sex difference was apparent in the case of children. A study by Diamant and Polishuk (1979) proposed the use of levels of leukocyte alkaline phosphatase determined cytochemically as an indicator of ovulation. They reported an increase to approximately five times the basal level upon ovulation, indicating that levels of leukocyte alkaline phosphatase vary substantially during the menstrual cycle. They suggested that early

in the cycle estrogens cause an increase in activity and that later progesterone inhibits the response of leukocyte alkaline phosphatase to the estrogens. Macaraeg et al (1968) reported that oral contraceptive agents also elevated leukocyte alkaline phosphatase scores. Levels have also been reported to fluctuate in situations of stress such as surgery, infection, or injection of adrenocorticotropin or steroid hormones (Valentine and Beck, 1951; Valentine et al, 1954). As previously stated the blood samples used in the present study came from donors of unknown age and sex so that wide variations might well be expected.

The inhibition patterns obtained for both the crude and purified extracts of alkaline phosphatase from granulocytes are identical to that of liver and are distinctly different from the intestinal and from the placental pattern.

The liver form of alkaline phosphatase is known to be L-phenylalanine stable, inhibited by urea and intermediate in terms of heat lability. The skeletal form is similar except for its marked heat lability. Conversely the placental form is inhibited by L-phenylalanine, only partially inhibited by urea and is heat stable. The intestinal form resembles the placental enzyme except that it has an intermediate heat lability.

Several studies have been performed on leukocyte alkaline phosphatase which allow its placement in the above scheme. Heat lability of leukocyte alkaline phosphatase has been detected in several studies (Bottomley et al, 1969; Tangheroni et al, 1971; Diamant et al, 1970; Findlay and Johnston, 1977; Wilson et al, 1981). Inhibition of enzyme activity in leukocytes by urea was reported by Tangheroni et al

(1971) and Findlay and Johnston (1977). Several reports have also been made of the stability of leukocyte alkaline phosphatase in the presence of L-phenylalanine (Tangheroni et al, 1971; Findlay and Johnston, 1977; Wilson et al, 1981). On the basis of all of these reports it is evident that the alkaline phosphatase present in leukocytes resembles the hepatic and skeletal forms of the enzyme, and behaves differently from the intestinal and placental forms. Findlay and Johnston (1977) claimed that the leukocyte enzyme was indistinguishable from the skeletal enzyme on the basis of electrophoretic studies. Although Wilson et al (1981) reported that the behaviour of alkaline phosphatase within the neutrophils was similar to that of the hepatic and skeletal forms, they also reported the detection of alkaline phosphatase activity on the external surface of the plasma membrane of the neutrophils which had properties characteristic of the placental isoenzyme. This is the only report to date of such activity. Certainly in the present investigation, no evidence of a mixed pattern of inhibition was exhibited which should have been the case if two isoenzymes were present in the extracts.

Based on studies of peptide mapping, immunological specificity and lectin-binding affinity (McKenna et al, 1979; Seargeant and Stinson, 1979; Sussman et al, 1968; Lehmann, 1980) it is now known that three structural genes code for alkaline phosphatase. The three gene products formed are the intestinal, liver/kidney/bone, and placental isoenzymes. When differential inhibition studies were used to detect the presence of alkaline phosphatase isoenzymes,

it was clearly indicated that granulocyte alkaline phosphatase is the product of the same gene which codes for the liver, kidney and bone forms of human alkaline phosphatase.

CHAPTER SIX

General Discussion

The majority of alkaline phosphatase activity of leukocytes was found within the neutrophil population in the present study. Unfortunately, conclusive confirmation of the presence of alkaline phosphatase within normal lymphocytes was not obtained since contamination of the lymphocyte population by neutrophils of approximately 5% on a cellular basis occurred. Nevertheless, it is valid to conclude that if the enzyme is present in lymphocytes, it is at very low levels. Since no evidence of a mixed pattern of inhibition was obtained when the contaminated lymphocyte population was studied with specific inhibitors, it would perhaps appear that if lymphocytes do normally contain alkaline phosphatase it is the same gene product as the enzyme present in neutrophils. However, the possibility of a different form of the enzyme being present in too low a concentration for detection cannot be ruled out. Studies of larger and purer lymphocyte populations are required to clarify this issue.

These studies could not substantiate the existence of an unique alkaline phosphatase which is incapable of cysteamine-S-phosphate hydrolysis and acts as a marker for lymphoproliferative disorders. Ectopic production of alkaline phosphatase has been detected in several neoplastic proliferative disorders and the enzyme produced has often resembled the placental form of the enzyme. In this study it was found that the placental form of alkaline phosphatase had the lowest K_m value for CASP of all the human multiple forms. Therefore, one might expect that an alkaline phosphatase from neoplastic tissue

would utilize CASP better than other forms of the enzyme.

On the other hand, the intestinal form of alkaline phosphatase had the highest K_m value for CASP. If a serum contained a large proportion of intestinal alkaline phosphatase and the hydrolysis of CASP was measured at a substrate concentration equal to the enzyme's K_m value (as done by Neumann et al, 1979) the rate of CASP hydrolysis would appear decreased. By contrast, if a CASP concentration which was saturating for the enzyme was used the rate of hydrolysis would appear normal. Perhaps the apparent inability of alkaline phosphatase in the sera of patients with lymphoproliferative disorders to hydrolzye CASP which was reported by Neumann et al (1979) was due to an increase of one particular form of the enzyme such as the intestinal, rather than the presence of an unique form of alkaline phosphatase. Further investigations are needed in order to assess the prevalence of alkaline phosphatase production in lymphoid neoplasms and to characterize the alkaline phosphatase in cases where it is produced.

The clinical importance of alkaline phosphatase activity in neutrophils has been recognized for several decades and a cytochemical stain for this activity has been part of the routine hematology methodology for many years. However, as previously mentioned the results using this type of method are semiquantitative and, perhaps more importantly, subjective. The future trend will likely be towards a more accurate and reproducible procedure such as a biochemical assay on a population of neutrophils as used in this investigation.

The method of granulocyte isolation used in this study involved a

dextran-mediated sedimentation of erythrocytes followed by centrifugation of the plasma on a discontinuous density-gradient. Both procedures are simple and are suitable for a routine procedure. The Wright-stained smears of the suspensions of isolated granulocytes revealed little contamination by either mononuclear leukocytes or erythrocytes which was supported by the good correlation between the protein values and granulocyte counts. Once neutrophil isolation is accomplished one can easily envisage adaptation of butanol extraction and assay of alkaline phosphatase activity to an automated procedure.

Based on the alkaline phosphatase activity in aqueous solutions of butanol extracts of granulocytes from fifty healthy individuals in this study, the mean ± 2 S.D. was 5.8 - 34.2 $mU/10^7$ granulocytes. Although this degree of variation is unusual for a reference range it is understandable in view of the influences of age and sex on the levels of neutrophil alkaline phosphatase.

It was found in the present investigation that each of the three known isoenzymes of human alkaline phosphatase could easily be identified through the use of only three inhibitors. Two points are apparent from these kinds of studies on granulocyte alkaline phosphatase in both a crude and purified form. Firstly, granulocytes appear to contain a homogeneous population of alkaline phosphatase since a mixed pattern of inhibition was not detected. Secondly, granulocyte alkaline phosphatase appears to be coded for by the same gene as the liver/kidney/bone group of human alkaline phosphatases.

The use of the inhibitors employed in the present study offers an easy, straightforward procedure for identification of different gene

products of human alkaline phosphatase. The most commonly employed procedures in the past have been the determination of heat stability, L-phenylalanine inhibition and electrophoresis which must be used in combination in order to clearly differentiate between the three gene product possibilities. By contrast, using the inhibitors, a clear-cut identification of each of the three types can be made on the basis of only four assays which is far less complex and time-consuming than the more popular methods.

The simplicity of the procedure employed in the present study makes the use of specific inhibitors attractive for such things as confirmation of variations in level, rather than form, of alkaline phosphatase in neutrophils in such conditions as pregnancy and chronic myeloid leukemia or identification of gene products of alkaline phosphatase which are produced in certain malignancies.

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APPENDIX I

Criteria for Diagnosis of Disorders Included in this Study

The diagnosis of each disorder included in this study was reached on the basis of the presence of the following findings as well as an appropriate clinical picture:

Lymphomas - based on pathological findings in lymph node biopsies. Further classification could be made on the basis of a "starry sky" appearance in Burkitt's lymphoma or the presence of Reed Sternberg cells in Hodgkin's disease.

Myeloma - the presence of a monoclonal protein, plasmacytosis of the bone marrow and appropriate radiological findings.

Cancers - based on pathological findings in tumour biopsies

Leukemias - based on findings as outlined in the FAB classification of acute leukemias (Bennett et al, 1976)

Infectious mononucleosis - the presence of a heterophil antibody.